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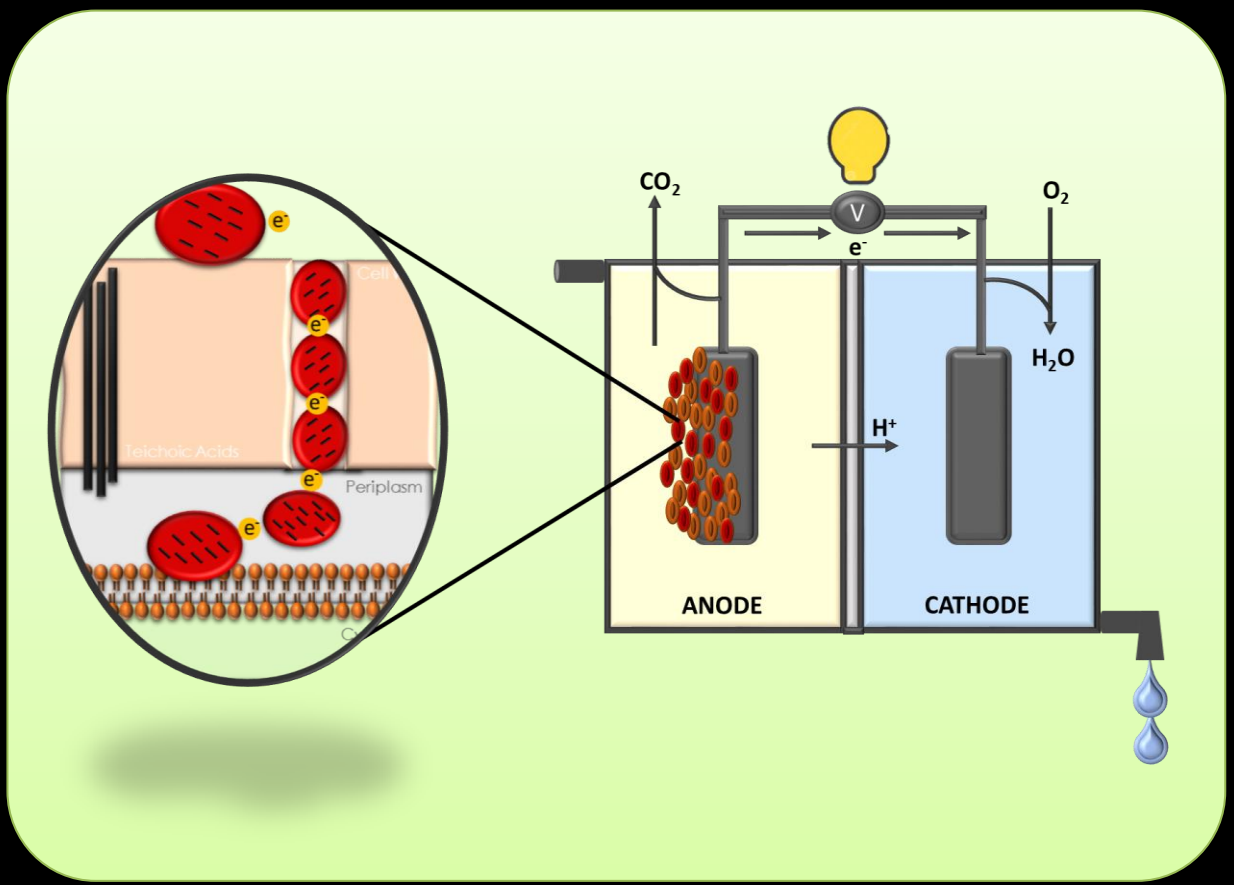
Nazua Lima Ferreira Costa



Dissertation presented to obtain the Ph.D degree in Biochemistry

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
December, 2017

UNIVERSIDADE NOVA DE LISBOA

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To my friends and family

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THESIS PUBLICATIONS

Published article

Costa, N.L., Hermann, B., Fourmond V., Faustino, M., Teixeira, M., Einsle, O., Paquete,C.M., Louro, R.O, *How thermophilic Gram-positive organisms perform extracellular electron transfer: characterization of the cell surface terminal reductase OcwA,* mBio **DOI: 10.1101/641308**

Nazua L. Costa, Thomas A. Clarke, Laura-Alina Philipp, Johannes Gescher, Ricardo O. Louro, Catarina M. Paquete *Electron transfer process in microbial electrochemical technologies: The role of cell-surface exposed conductive proteins,* Bioresource Technology 255, 308-317 (2018) **DOI: 10.1016/j.biortech.2018.01.133**

Costa, N. L., Carlson, H. K., Coates, J. D., Louro, R. O. and Paquete, C. M. *Heterologous expression and purification of a multiheme cytochrome from a Gram-positive bacterium capable of performing extracellular respiration,* Protein Expr. Purif. 111, 48–52 (2015). **DOI: 10.1016/j.pep.2015.03.007**

Published abstract

Costa N. L., H. K. Carlson, J. D. Coates, R. O. Louro, C. M. Paquete: *Improving the heterologous expression of cytochromes from Gram-positive bacterium Thermincola potens JR*
Abstract selected for Speedy Talk (ST-02.02.2-006) published on 41st FEBS Congress, Molecular and Systems Biology for a Better Life, Ephesus/Kuşadasi, Turkey, September 3-8, (2016)
http://onlinelibrary.wiley.com/doi/10.1111/febs.13807/epdf

OTHER PUBLICATIONS

Published article

Alexandra S. Alves, **Nazua L. Costa**, Ming Tien, Ricardo O. Louro and Catarina M. Paquete, *Modulation of the reactivity of multiheme cytochromes by site-directed mutagenesis: moving towards the optimization of microbial electrochemical technologies* **DOI: 10.1007/s00775-016-1409-0**

Published book chapter

Ricardo O. Louro, **Nazua L. Costa**, Ana P. Fernandes, Ana V. Silva, Inês B. Trindade, Bruno M. Fonseca and Catarina M. Paquete, *Exploring the molecular mechanisms of extracellular electron transfer for harnessing reducing power in METs*, book on 'Microbial Electrochemical Technology: Platform for Fuels, Chemicals and Remediation'. This book is a part (Volume I) of a new series on 'Biomass, Biofuels and Biochemicals' to be published by Elsevier

ABREVIATIONS

AQDS- Anthraquinone – 2,6 – disulfonate

c-Cyts – Cytochrome *c*

DRMB – Dissimilatory Metal Reducing Bacteria

DET – Direct Electron Transfer

EET – Extracellular Electron Transfer

EPR – Electron Paramagnetic Resonance

HAO - Hydroxylamine oxidoreductase

FMN – Flavin Mononucleotide

Fcc3 – Fumarate reductase flavocytochrome c_3 from *Shewanella oneidensis* MR1

IET – Indirect Electron Transfer

MacA – cytochrome *c-551* peroxidase

MccA – Sulfite reductase form I

MET – Microbial Electrochemical Technologies

MFC – Microbial Fuel Cells

MHC – Multiheme *c*-type Cytochrome

MES – Microbial Electrochemical Systems

MtrA – Metal terminal reductase subunit A

MtrB - Metal terminal reductase subunit B

MtrC - Metal terminal reductase subunit C

NMR – Nuclear Magnetic Ressonance

NrfA – cytochrome *c*-552

OcwA – Outer cell wall protein A

OmcA – Outer membrane cytochrome A

OmcB – Outer membrane cytochrome B

OmcE – Outer membrane cytochrome E

OmcS – Outer membrane cytochrome S

PMS – Phenazine Methosulphate

PpcA – Periplasmic *c*-type triheme cytochrome

STC – Small Tetraheme Cytochrome *c*

PEM – Proton Exchange Membrane

RESUMO

As Tecnologias Eletroquímicas Miicrobianas (METs) estão hoje em dia no foco da investigação aplicada e fundamental. As pilhas de combustível microbianas (MFCs) são um exemplo destas biotecnologias emergentes que combinam processos biológicos e eletroquímicos para a produção de eletricidade, hidrogénio ou de outros produtos do valor acrescentado. Estes dispositivos dependem da interação elétrica entre bactérias ambientalmente ubíquas e elétrodos dedicados. Apesar do seu potencial inerente para a produção de energia sustentável e o tratamento de águas residuais, as MFCs ainda são pouco aplicadas em processos industriais. Isto deve-se principalmente à baixa eficiência de transferência de eletrões entre as bactérias e os elétrodos. Compreender os mecanismos moleculares subjacentes à transferência de eletrões na interface bactérias-elétrodos é, portanto, crucial para melhorar a eficiência deste processo e acelerar a aplicação prática das MFCs.

Atualmente, o conhecimento sobre a transferência extracelular de eletrões (EET) deriva dos extensos estudos feitos em bactérias mesofílicas Gram-negativas que mostraram que os citocromos multihémicos de tipo *c* (MHC) são os principais intervenientes neste processo. Recentemente, bactérias termófilas Gram-positivas têm vindo a ser isoladas a partir do ânodo de MFCs que operam a elevadas temperaturas (acima de 55ºC). Estas bactérias termófilas demonstraram serem capazes de gerar mais eletricidade do que as bactérias Gram-negativas mesofílicas no mesmo tipo de reatores, sendo na maioria das vezes a espécie prevalente nos consórcios microbianos. Devido à ausência de uma membrana externa, à presença de uma parede celular espessa (10 a 80 nm) e uma camada de glicoproteína S, as bactérias

Gram-positivas foram consideradas incapazes de transferir eletrões para os aceitadores terminais insolúveis. No entanto, a sequenciação do genoma destas bactérias electroactivas termofílicas revelou a existência de vários genes que codificam para MHCs, sugerindo o contrário.

Este trabalho tem como objectivo perceber as bases moleculares sobre as quais uma das bactérias termófilas Gram-positivas isoladas recentemente, *Thermincola potens* JR, realiza EET. Para tal, as proteínas com papéis fundamentais neste processo precisam ser estrutural e funcionalmente caracterizadas. *T. potens* JR é uma bactéria altamente desafiadora para cultivar em regime laboratorial devido à sua taxa de crescimento lento e baixa densidade óptica. Por outro lado, *E. coli* é o sistema de expressão ubíquo para proteínas heterólogas e eficiente na produção de MHCs. Utilizando ferramentas de biologia molecular, desenvolveu-se uma nova estratégia para expressar e purificar heterologamente os MHCs de *T. potens* JR em *E. coli*. A produção de citocromos de tipo *c* necessita de máquinas celulares específicas, tais como o sistema de translocação Sec que transporta apo-proteínas do citoplasma para o espaço periplásmico através do reconhecimento de regiões específicas das sequências de péptidos de sinal. Enquanto *E. coli* usa o sistema Sec B como o principal sistema de translocação, *T. potens* JR usa o sistema Sec A. Assim, para assegurar o reconhecimento e translocação dos MHCs de *T. potens* JR pela *E. coli*, foi construído um gene quimérico. Este gene foi obtido por fusão do peptídeo sinal do citocromo *c* tetrahémico (STC) da bactéria Gram-negativa *Shewanella oneidensis* MR-1, com a sequência do gene de proteínas potenciais de *T. potens* JR sem o seu péptido sinal nativo. Utilizando esta metodologia, o citocromo periplásmico decahémico TherJR_0333 e o citocromo

nonahémico de superfície celular OcwA de *T. potens* JR foram sobre-expressos com sucesso e purificados a partir de *E. coli*.

TherJR_0333 foi o primeiro MHC Gram-positivo alguma vez expresso e purificado heterologamente a partir de um sistema de expressão Gram-negativo. A proteína recombinante solubilizada revelou o espectro de UV-visível típico de um citocromo de tipo *c* hexa-coordenado de spin baixo, tanto nas formas oxidadas como reduzidas. Por seu lado as experiências de NMR também confirmaram a presença de hemos de spin baixo, no qual os picos dos grupos metilo surgem caracteristicamente deslocados para a região paramagnética de campo baixo, até 40 ppm. A espectrometria de massa mostrou que a proteína recombinante continha dez hemos.

O citocromo de superfície celular OcwA é uma proteína de nove hemos que se prevê que seja a redutase terminal de metais. A estrutura tridimensional desta proteína juntamente com os dados EPR permitiu a identificação de três conjuntos de hemos com diferentes tipos de coordenação: bis-His (I, III, IV, VI, VII e VIII), His-Met (IX) e duas His distaais não-ligadas (II e V). A estrutura também revelou que a arquitetura dos hemos é distinta do arranjo de "cruzamento escalonado" observado para os citocromos de membrana externa das bactérias mesofílicas Gram-negativas extensivamente estudadas, sugerindo um comportamento funcional diferente.

O EET realizado por bactérias eletroativas pode ocorrer através do contato direto entre as bactérias e o elétrodo ou indiretamente usando pequenas moléculas redox ativas como transportadoras de eletrões. Estudos anteriores sugerem que *T. potens* JR é dependente de contacto direto para a ocorrência do processo de transferência de eletrões para substratos extracelulares. No entanto, as experiências de ligação

seguidas por NMR revelaram que o OcwA também é capaz de interagir com transportadores solúveis de electrões tais como as flavinas. Esta interação também foi confirmada através de estudos cinéticos nos quais outros transportadores solúveis de eletrões, tais como riboflavina, antraquinona-2,6-dissulfonato (AQDS) e fenazina metossulfato (PMS) também foram reduzidos por esta proteína.

As bactérias electroactivas termofílicas são candidatas muito promissoras para serem utilizadas em MFCs de alta temperatura devido à sua capacidade de manter uma actividade microbiana aumentada, uma melhor solubilidade dos substratos, uma taxa de transferência de massa elevada e um menor risco de contaminação com espécies microbianas não termófílicas. Perceber os mecanismos moleculares pelos quais as bactérias termófilas eletroativas realizam EET é crucial para entender a diversidade metabólica que governa os ciclos redox ambientais e para otimizar as aplicações que dependem desses microrganismos. Nesse sentido, a metodologia para expressão heteróloga de MHCs de bacterias Gram-positivas desenvolvida neste estudo além de ser pioneira também abre a possibilidade de caracterizar uma série de outras proteínas de bactérias Gram-positivas. Além disso, este conhecimento acabará por orientar o uso racional desta classe promissora de bactérias termófilas para a aplicação prática de dispositivos bioeletroquímicos. Estes dispositivos representarão um passo importante para um ciclo fechado de tratamento de resíduos orgânicos e produção de energia sustentável.

ABSTRACT

Microbial Electrochemical Systems (METs) are nowadays in the focus of applied and fundamental research. Microbial fuel cells (MFCs) are an example of these rapidly emerging biotechnologies that combine both biological and electrochemical processes towards the generation of electricity, hydrogen or other added value products. These devices rely on the electrical interaction between environmentally ubiquitous bacteria and dedicated electrodes. Despite their inherent potential for sustainable energy production and wastewater treatment, MFCs are still poorly applied in industrial processes. This is mainly due to the low electron transfer efficiency between bacteria and electrodes. Understanding the molecular mechanisms underlying the electron transfer at the bacteria-electrode interface is therefore crucial to improve the efficiency of this process and accelerate the practical application of MFCs.

Currently, the knowledge on extracellular electron transfer (EET) derives from extensive studies on Gram-negative mesophilic bacteria which showed that multiheme *c*-type cytochromes (MHCs) are key players in this process. Recently, Gram-positive thermophilic bacteria were isolated from the anode of MFCs operating at high temperature (above 55^{o}C). These electroactive bacteria were shown to generate more current than mesophilic Gram-negative bacteria in the same type of reactor, being most of time the prevalent species in microbial consortia. Due to the lack of an outer membrane and the presence of a thick cell wall (10 to 80 nm) and a glycoprotein S layer, Gram-positive bacteria were considered incapable of direct electron transfer to insoluble electron acceptors. However, genome sequencing of these thermophilic

electroactive bacteria revealed the existence of several genes that code for MHCs, suggesting otherwise.

This work focuses on unraveling the molecular bases by which one of the recently isolated Gram-positive thermophilic bacteria, *Thermincola potens* JR, performs EET. In order to do so, the proteins with key roles in this process need to be structurally and functionally characterized. *T. potens* JR is a highly challenging bacterium to cultivate in large scale due to its slow growth rate and low optical density. On the other hand, *E. coli* is the ubiquitous expression system for heterologous proteins and efficient in the production of MHCs. Using molecular biology tools, a novel strategy to heterologously express and purify the MHCs from *T. potens* JR in *E. coli* was developed. The production of *c*-type cytochromes requires specific cell machinery, such as the Sec translocation system that transports apo-proteins from the cytoplasm to the periplasmic space through the recognition of specific regions of the signal peptide sequences. While *E. coli* uses Sec B as the major translocation system, *T. potens* JR uses Sec A. Thus, to ensure the recognition and translocation of *T. potens* JR MHCs by *E. coli*, a chimeric gene was constructed. This gene was obtained by fusing the signal peptide from the small tetraheme cytochrome *c* (STC) from the Gram-negative bacterium *Shewanella oneidensis* MR-1, with the gene sequence of *T. potens* JR target proteins without their native signal peptide. Using this methodology, the decaheme periplasmic cytochrome TherJR_0333 and the nonaheme outer-cell surface cytochrome TherJR_2595 from *T. potens* JR were successfully over-expressed and purified from *E. coli*.

TherJR_0333 was the first Gram-positive MHC ever expressed and purified heterologously from a Gram-negative expression system. The

solubilized recombinant protein revealed the typical UV–visible spectrum of a low spin hexa-coordinated *c*-type cytochrome in both oxidized and reduced forms. While NMR experiments confirmed the presence of low spin hemes, where the peaks from the methyl groups are shifted to the paramagnetic low field region up to 40 ppm, mass-spectrometry showed that the recombinant protein contained ten hemes. The outer-cell surface cytochrome TherJR_2595 is a nine-heme protein predicted to be the putative terminal metal reductase. The three-dimensional structure of this protein together with EPR data allowed the identification of three set of hemes with different kinds of heme coordination: bis-His (I, III, IV, VI, VII and VIII), His-Met (IX) and His+ distal non-bonded di-His (II and V). The protein structure also revealed that the architecture of the heme core is distinct from the "staggered cross" arrangement observed for the outer-membrane cytochromes from the extensively studied Gram-negative mesophilic bacteria, suggesting a different functional behavior.

The EET performed by electroactive bacteria can either occur through direct contact between bacteria and the electrode or indirectly by using small redox molecules as electron shuttles. Previous studies suggest that *T. potens* JR is direct-contact dependent regarding the electron transfer to extracellular substrates. However, NMR binding experiments revealed that TherJR_2595 is also able to interact with electron shuttles such as flavins. This interaction was also confirmed through fast kinetic techniques where electron shuttles such as riboflavin, antraquinone-2,6-disulfonate (AQDS) and phenazine methosulphate (PMS) were also reduced by this protein.

Thermophilic electroactive bacteria are highly promising candidates to be used in high temperature MFCs due to their ability to maintain

increased microbial activity, better substrate solubility, high mass transfer rate and lower risk of contamination with non-thermophilic microbial species. Unraveling the molecular mechanisms by which thermophilic electroactive bacteria perform EET is crucial to understand the metabolic diversity that rules the environmental redox cycles and to optimize applications relying on these microorganisms. In this sense, the methodology for heterologous expression of Gram-positive MHCs developed in this study besides being pioneer also opens the possibility to further characterize cytochromes from other Gram-positive bacteria. Furthermore, this knowledge will ultimately guide the rational use of this promising class of thermophilic bacteria towards the practical application of bioelectrochemical devices. These devices will represent a major step towards a closed cycle of organic residues treatment and sustainable energy production.

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OVERVIEW

The work presented in this thesis is divided in five chapters. In Chapter I the state of the art is reviewed comparing the extracellular electron pathways widely known in Gram-negative bacteria with the proposed model for *T. potens* JR. This chapter also includes explanation about electroactive microorganisms and their biotechnological application.

In Chapter II the methodologies that were employed for the development of the practical work are discussed in an attempt to provide the reader with all the necessary tools to follow the scientific approaches presented in the following chapters.

Chapter III is a transcript of a publish article that describes the successful production of the first multiheme *c*-type cytochrome from a Gram-positive bacterium, *T. potens* JR, using Gram-negative bacteria as expression system. This chapter also includes the biochemical characterization of the produced recombinant protein.

Chapter IV is an article in preparation where the full functional and structural characterization of the first cell surface multiheme *c*-type cytochrome from Gram-positive bacteria is described. The work presented in this chapter was performed in collaboration with Dr. Oliver Einsle and Dr.Bianca Hermann from the University of Freiburg, which kindly solved the structure of the surface exposed protein; Dr. Christophe Léger and Dr. Vincent Formound from BIP6 Aix-Marseille, which received me on their lab to perform the electrochemical characterization of the protein and Dr. Miguel Teixeira with whom I performed the EPR experiments.

On the concluding Chapter V, an overall discussion of the work is made pointing out some results that although not shown on the previous

chapters, were crucial for their progress. Ideas for future work are also discussed. This chapter ends with some highlights on the importance of thermophiles like *T. potens* JR towards the practical application of biotechnologies that use microorganisms to harvest environmentally friendly energy and wastewater treatment.

Chapter I

General Introduction

INTRODUCTION

Over the past 100 years the Earth´s climate has warmed approximately 1.7ºC and recent studies forecast a temperature rise of 0.9ºC to 5.6ºC over the next century[1]. This gradual global warming is a result of industrial revolution that took place in the beginning of the XVIII century reaching its peak in the mid 1970´s [1]. By that time several highly contaminant compounds such as coal, copper, lead, some radioactive species and fossil fuels were used as power sources to run the industrial machines, as well as raw materials to fabricate all types of valuable products. The continuous emission of CO_2 to the atmosphere as a result of the widespread industrial activity lead to dramatic increase of the greenhouse effect which is responsible for the severe climate changes from the early begining of the twenty century, including rising of Earth's temperature [2].

The direct impact of climate changes has urged the need to establish adequate commitments towards the reduction of carbon emissions and the development of alternative energy sources [3–5]. Over the past decades the production and consumption of alternative energy sources with low ecologic footprint such as solar, wind, geothermal and biofuels have significantly increased. It is in this context that Microbial Electrochemical Technologies (MET) have also emerge as a sustainable alternative for energy production and wastewater treatment [6, 7]. This technology uses environmentally ubiquitous microorganisms as catalysts to convert the chemical energy stored in reduced organic compounds, like municipal waste, to produce electrical current, hydrogen and several added value compounds [8–10].

[1] https://climate.nasa.gov/effects_21.09.2017

Currently, the applications of MET go further than the typical Microbial Fuel Cells (MFC) in which the chemical energy contained in low-cost organic matter is recovered directly as electrical energy or the Microbial Electrolysis Cells (MEC) that requires an external electrical current to drive the reduction process towards the production of hydrogen and other added value compounds [11, 12]. By taking advantage of the unique features of these systems, which is the ability to provide both oxidation (anode) and reduction (cathode) process at the same time, a plethora of METs systems with different architectures and characteristics that can be used for numerous technologies are emerging [13–16].

However, under the scope of this dissertation only MFC will be considered with particular focus on the microorganisms that can be used on the anode compartment.

1.1. MICROBIAL FUEL CELLS

MFC are generally composed by two compartments: the anodic and the cathodic chamber. (Figure I.1).

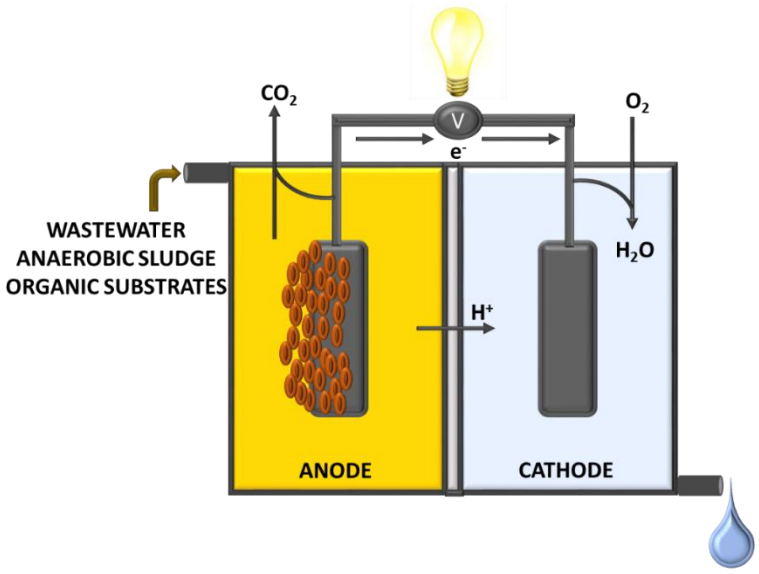

Figure I.1: Schematic representation of the electron flow in a typical double chamber MFC

In these devices, microorganisms grow as biofilm on the surface of the anode by oxidizing organic matter available in the medium (that can be domestic or industrial wastewater), and convert it into CO_2, electrons and protons [11]. While the electrons resulting from the bacterial metabolism are transferred to the anode, CO_2 and protons are released into the electrolyte solution. On a typical double chamber MFC, the anode is separated from the cathode by a proton exchange membrane (PEM). Both electrodes are connected to each other by a resistor that imposes a potential difference allowing the electrons to travel through the electrical circuit generating energy. The circuit is closed at the cathode compartment where the oxygen is combined with the protons that cross the PEM and electrons to produce water (Figure I.1) [11].

The microorganisms that in the presence of an electrode have the ability to transfer to it the electrons resulting from their metabolism or vice versa are designated as electroactive [17, 18].

1.2. ELECTROACTIVE MICROORGANISMS

The ability of some microorganisms to generate electrical current while degrading organic compounds was first reported by Potter in the beginning of the twenty century [19]. Currently, more than 90 species of electroactive microorganisms have been isolated from natural environment or in MFC consortia [20]. Several electroactive species are also considered dissimilatory metal reducing bacteria (DMRB) due to their they ability to reduce insoluble metals such as: Fe(III), Mn(IV), Cr(VI), U(VI), Tc(VII), Co(III), Mo(VI), Hg(II) [21–23] and Au (I, III) [24] while performing their anaerobic respiration.

The DMR ability was originally discovered in bacteria belonging to *Geobacter* spp. and *Shewanella* spp. [25, 26] and still to date *Geobacter sulfurreducens* PCA and *Shewanella oneidensis* MR1 are the most well characterized DMRB. Due to their ability to efficiently transfer electrons to the anode in a MFC with a high columbic efficiency [27, 28], they have become the model organisms for investigating EET in MFCs. Recently, the thermophilic Gram positive bacterium *Thermincola potens* strain JR was isolated from a biofilm community formed on the anode of an acetate-fed MFC inoculated with methanogenic anaerobic sludge (55ºC) [29]. Studies on the bacterial content of the anodic community revealed that *T. potens* JR was the dominant species and the major electricity producing strain [29]. Pure culture MFC studies showed that *T.potens* JR can generate a current of 0.42mA with a columbic efficiency of 91% using acetate as electron donor [30]. This was the first time that a Gram-positive bacterium was demonstrated to efficiently transfer electrons to the anode of a MFC generating electrical current.

Furthermore, some studies on MFC revealed that thermophilic Gram-positive bacteria were able to generate more electrical current than the representative mesophilic Gram-negative bacteria in the same type of reactor [27–29].

1.3. ADVANTAGE OF THERMOPHILIC MFC

Thermophilic anaerobes are usually linked to the early forms of life due to their ability to inhabit extreme environments like underwater hydrothermal vents, hot springs and geysers where higher temperatures, pressure and anoxic conditions are a natural imposition [31, 32]. Indeed, several thermophilic anaerobes were also shown to be able to reduce various metals like Fe(III), Mn(IV), Cr(VI), U(VI), Tc(VII), Co(III), Mo(VI), Au (I, III), and Hg(II) [33]. Fe(III)-reducing thermophiles were found in almost all thermophilic environments [34]. Among them, Gram-positive bacteria belonging to the phylum Firmicutes appear to predominate, representing about 60% of the overall diversity of thermophilic Fe(III) reducers reported so far [33, 34]. DMRB from the genus *Thermincola* belong to this phylum and are nowadays under scientific scrutiny due to their high performance on MET [29, 36–38]. Additionally, thermophilic microorganisms bring numerous advantages in operating MFC, such as:

i) potentially higher energy yields due to the temperature increasing in the system that allows improvements in electron production rates;

ii) easier maintenance of anaerobic reducing conditions because of the lower solubility of O_2 at elevated temperatures; and if it is desirable maintenance of pure cultures.

All these features make thermophilic Gram-positive bacteria excellent candidates for wastewater treatment and energy production in MFC. Nevertheless, towards their practical application it is necessary to understand the mechanisms underlying the EET process in these promising class of electroactive bacteria.

1.4. ROLE OF MHC IN EET

Extensive studies on the electroactive model organisms, *Geobacter sulfurreducens* PCA and *Shewanella oneidensis* MR-1, have led to the identification of two general mechanisms for EET: direct electron transfer (DET) and indirect electron transfer (IET). In DET the bacteria establish direct contact with insoluble substrates or electrodes via outer membrane *c*-type cytochromes (*c*-Cyts) or through cellular appendages such as pili or nanowires, that were shown to be also associated with *c*-Cyts. In IET bacteria use exogenous or endogenous soluble redox active compounds as electron shuttles to mediate electron transfer between outer membrane proteins and insoluble electron acceptors (Figure I.2) [39] Notwithstanding, both mechanism are linked to the presence of redox active multiheme *c*-Cyts (MHC) that are key players in EET process [40–42]. In fact, 111 genes coding for *c*-Cyts were found in *G. sulfurreducens* PCA of which 72 coding for proteins with more than two heme groups. So far, this is the highest amount of *c*-Cyts found in electroactive bacteria [43]. From the 42 genes that code for *c*-Cyts in *S. oneidensis* MR1 33 have more than one heme group and are known to participate in the various respiratory pathways of this bacterium being linked to its capacity to use a wide variety of terminal electron acceptors [44].

Interestingly, genome sequencing of *T. potens* JR showed the presence of 33 genes coding for MHC [45] with an average of approximately 12 hemes per *c*-Cyt [30]. The analysis of *T. potens JR* growing medium revealed that this bacterium does not excrete soluble redox mediators thus requiring only direct contact with the solid substrate for electron transfer to occur [30].

1.5. EET PATHWAY IN GRAM-NEGATIVE BACTERIA

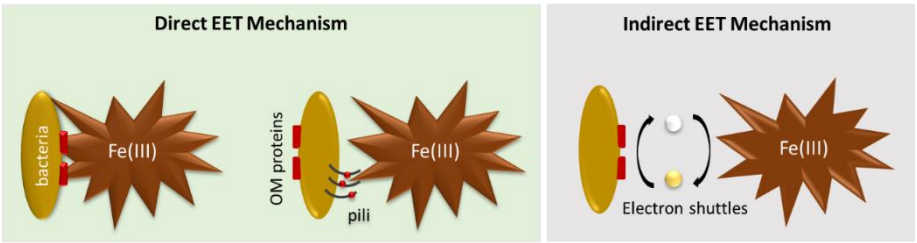

Figure I.2: Extracellular electron transfer mechanisms (adapted from Nevin and Lovley 2002)

To date it is well establish that a wired like arrangement of MHC is crucial to enable metabolic electrons to be transferred from the inner membrane to the cell surface [46–48].

The electroactive *S. oneidensis* MR-1 and *G. sulfurreducens* are known to rely on "porin-cytochrome" complexes that establish a relay to transfer electrons from the cytoplasmic membrane to the periplasmic space and to the outer-membrane toward extracellular electron acceptors [49].

In *S. oneidensis* MR-1 the EET starts with the inner membrane bound tetraheme cytochrome CymA that delivers electrons from the quinone pool to periplasmic *c*-Cyt, including the small tetraheme cytochrome *c* (STC) and the fumarate reductase flavocytochrome c_3 (Fcc3). These two cytochromes exchange electrons with the periplasmic decaheme

cytochrome MtrA which than transfers them to the outer membrane decaheme cytochrome complex MtrC/OmcA [49, 50]. Both MtrA and MtrC are inserted in MtrB which is a β-barrel porin forming the MtrCAB complex. This complex span ~40 Å of the outer-membrane of *Shewanella*, mediating the transfer of electrons from one side of the lipid bilayer to the other through a chain of 20 hemes that is formed between the two cytochromes (Figure 1.3A). The three proteins in this complex were shown to be functionally dependent from each other [50, 51], being MtrA crucial protein for the assembly of the MtrCAB complex and stabilization of the MtrB porin [50].

The decaheme cytochromes OmcA and MtrC are considered the terminal metal reductase in *Shewanella spp*, being responsible for both direct and indirect electron transfer. It was proposed that MtrC receives electrons from the periplasmic space via MtrA and then transfers them to insoluble terminal electron acceptors, electron shuttles, or to OmcA [44, 52–55]. Deletion mutant strains of both MtrC and OmcA display serious deficiency in the reduction of extracellular metallic compounds [56, 57]. Moreover, it was recently demonstrated that these two outer membrane cytochromes are major components of the electrically conductive nanowires found in *S. oneidensis* MR-1, which were shown to be membrane rather than pilin based structures [58, 59].

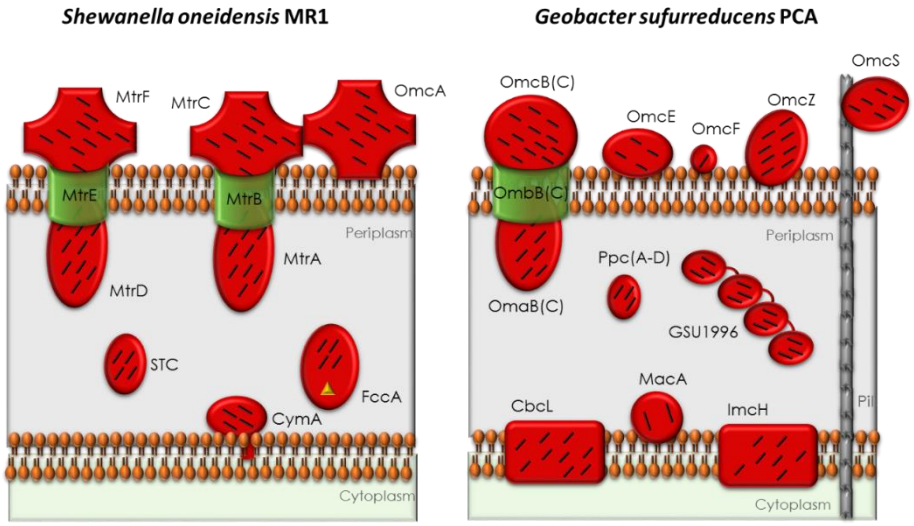

Figure I.3: Schematic representation of the EET pathways of A) *S.oneidensis* MR1 and B) *G. sulfurreducens*

Although several *c*-Cyts essential for EET have been identified in *G. sulfurreducens* PCA the exact mechanisms performed by this organism remains to be elucidated. Notwithstanding, it was shown that the PpcA and the outer membrane OmcB, OmcC and OmcS are important for the electron transfer to Fe (III) [60]. The inner membrane diheme peroxidase MacA was found to have no Fe(III) reduction ability [61], yet it mediates the electron transfer from the quinone pool to PpcA [62]. The outer-membrane dodecaheme cytochromes OmcB and OmcC belong to a trans outer membrane porin complex that also includes porin-like outer-membrane proteins (OmbB and OmbC) and periplasmic *c*-Cyts (OmaB and OmaC) [63], in a protein arrangement similar to the MtrCAB complex from *S. oneidensis* MR-1 (Figure I.3B). Indeed, homologues of this complex can be found in eight sequenced *Geobacter spp.*, and in other bacteria including sulfate reducing bacteria [63].

OmcS and OmcE, are other *c*-Cyts found in the outer-membrane of *G. sulfurreducens* which are loosely attached to the cell surface, and were shown to be important for the reduction of insoluble Fe(III) oxides [64]. Of these, OmcS, besides being one of the most abundant outer-membrane *c*-Cyt, is one of the most interesting as it is associated with pili [65]. It was proposed that OmcS facilitates the last step of Fe(III) oxide reduction acting as a freeway for the fast electron transfer along the conductive pili [65, 66] being also is also crucial for the electron transfer to electrodes and for the reduction of humic substances [67, 68].

1.6. EET PATHWAY IN GRAM-POSITIVE BACTERIA

Unlike Gram-negative bacteria, the cell envelope of Gram-positive bacteria is characterized by the lack of an outer membrane, a thick cell wall (10 to 80nm), a narrow periplasm (~10nm) filled with low density proteins and sometimes the presence of a glycoprotein S layer. These bacteria also have teichoic acids imbedded in their cell wall, which can extend from the inner membrane to the outer surface of the peptidoglycan layer resulting in a dense and crowded structure [69–72]. For that reason, the Gram-positive cell envelope was for several years considered incompatible with an EET pathway supported on a wired like network of redox proteins.

Interestingly, several biochemical techniques coupled with Raman spectroscopy and Microscopy were used to determine the subcellular localization of some of the MHC coded in *T. potens* JR genome leading to the proposal of the first hypothetical EET pathway for a Gram-positive bacterium (Figure I.4) [73].

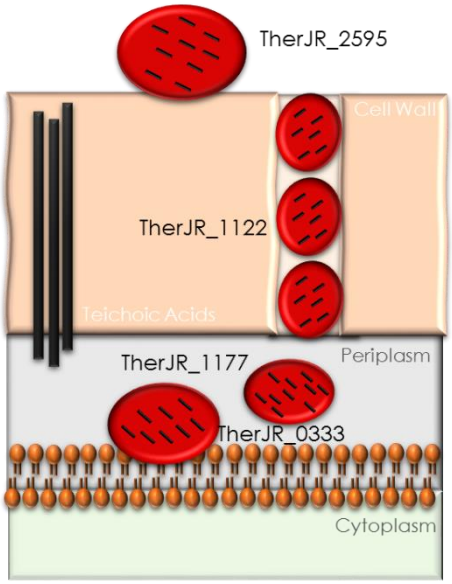

Figure I.4: Hipothetical extracelullar electron transfer pathway of *T.Potens JR* (adapted from Carlson et. al PNAS 2011)

As can be seen, the *T. potens* JR EET pathway resembles the MHC wired like network proposed for Gram-negative bacteria. In this model, the decaheme inner membrane bound protein TherJR_1117 is the quinol dehydrogenase that transfers the electrons from the quinol pool to the decaheme periplasmic protein TherJR_0333. This in turn transfers the electron to the cell wall embed hexaheme TherJR_1122, which due to its molecular weight of 41kDa is likely to form a wire of individual proteins along the peptidoglycan wall. The final step is the electron transfer to the putative terminal metal reductase nine heme TherJR_2595.

Unlike the proteins from the Gram-negative porin-cytochrome complexes, the genes that code for the periplasmic protein, the cell wall imbedded protein, and the surface exposed protein are not in the same operon, as can be seen for their gene annotation: *therjr_0333*, *ther_jr1122* and *ther_jr2595* respectively. This different locus

localization is consistent with the lack of a porin-like structure that bridges the terminal metal reductase with the periplasmic protein presented in this hypothetical model. Furthermore, it supports the idea that TherJR_2595 is attached to the cell surface.

Moreover, this model proposes that TherJR_2595 receives electrons from TherJR_1122. However, it is not clear how both proteins interact to ensure the electron transfer flow. In addition, alternative routes involving periplasmic hydrogenases were proposed for the initiation of the electron transfer process [73].

Other Gram-positive bacteria from the genus *Thermincola* were isolated from anaerobic microbial communities in terrestrial hot springs and classified as electroactive [74, 75]. In particular *Thermicola ferriacetica* was shown to grow at the expense of Fe (III) reduction and to form thick biofilms on the anode of MFC with high columbic efficiency [37]. This bacterium shares 99% similarity [76] with *T. potens* JR but the mechanisms by which it performs EET remains also to be unraveled.

Considering that, the work presented on this thesis was a crucial opportunity to shed light on the molecular bases by which a thermophilic Gram-positive bacterium performs EET. Understanding how the proposed MHC from *T. potens* JR interact with each other to ensure the electron transfer towards the terminal electron acceptors will reveal for the first time information on the mechanism for electron transport across the cell wall of Gram-positive bacteria. To achieve this goal, in vitro reconstitution and characterization of the proteins proposed to be involved in bioenergetic chain of *T. potens* JR was set as the focus.

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Chapter II

Methodology and Techniques

To date *Thermincola potens* JR is not genetically tractable and its manipulation on laboratory context is still difficult. To fulfill the aim of this thesis, several biological and biochemical approaches were pursued towards the target protein expression and characterization. In this section, the techniques that were crucial for the development of the work presented throughout the chapters III and IV are discussed in an attempt to contextualize the reader and to call its attention to the challenges of the fundamental study of the target proteins that are relevant for the extracellular electron transfer processes of *T. potens* JR.

OVEREXPRESSION OF MHC

Understanding the molecular bases that underlie EET requires the functional and structural characterization of the proteins that are key players in this process. Toward this objective, high quality protein in sufficient amounts are required. However, for the vast majority of electroactive microorganisms sufficient amounts of proteins are often hard to obtain due to slow growth rate of the cells or *c*-Cyts maturation. Considering that, recombinant production of MHC using appropriate bacterial expression systems is a desirable approach to meet these challenges.

Over the years, *E. coli* has been used as the preferential expression system for recombinant proteins. This bacterium holds several biotechnological advantages such as: rapid grow on inexpensive substrates achieving high cell density; fully sequenced genome and characterized metabolic pathways; and availability of an increasing high number of cloning vectors and mutant host strains [1–3].

The expression of *c*-Cyt requires a specific machinery that ensures the covalent attachment of heme *b* (Fe-protoporphyrin IX) to the apo-

cytochrome by the typical Cys-X-X-Cys-His (CXXCH) heme binding motif. In Gram-negative bacteria like *E. coli* the biosynthesis of *c*-Cyt is mediated by System I or the Ccm system that is located on the periplasmic side of the inner membrane. This complex enzymatic system usually involves eight proteins, the CcmA-H, that ensure the maturation of *c*-Cyt. In this complex, the CcmF is the *c*-Cyt heme lyase which is responsible to catalyze the covalent attachment of the heme to the apo-cytochrome. This is made via thioether bonds between two vinyl groups of the heme and two cysteinyl residues from the heme binding motif in the apo-protein [4, 5]. Since the heme assembly occurs in the periplasm, a dedicated secretion system to ensure the translocation of the unfolded apo-cytochrome from the cytoplasm to the periplasmic space is often required. In Gram-negative bacteria this role is played by the chaperon SecB [6–8].

The development of the pEC86 plasmid which contains the genes of the Ccm system allowed the production of *c*-Cyt during aerobic grow in *E. coli* [9]. Indeed, co-transformation of *E.coli* cells with this plasmid not only increased the protein yield but also ensured the reliability of the expression [10–12]. Furthermore, it made possible the heterologous expression of MHC [13–17].

Alternatively, *Shewanella oneidensis* MR-1 can also be used as an expression system for MHC [18, 19]. Unlike *E. coli*, *S. oneidensis* MR-1 has a native Ccm system which produces MHC in both anaerobic and aerobic conditions [20]. The pBAD202/D-TOPO plasmid from Invitrogen (Carlsbad, CA, USA) has been widely used for expression of MHC in both *S. oneidensis* MR-1 and in *E. coli* [21–24]. This plasmid is tightly regulated and allows high levels of protein expression through the inexpensive inducer arabinose. It also enables the incorporation of a

V5-epitope and a His6 tag at the C terminus of the expressed proteins that facilitate subsequent protein detection and isolation.

OVEREXPRESSION OF GRAM-POSITIVE MHC – THE CHALLENGE

Until the work reported on chapter III, the only *c*-Cyt expressed from a Gram-positive bacterium was the monoheme c_{553} from *Bacillus pasteuri*. This was made by double transformation of *E. coli* with pEC86 and the plasmid containing the c_{553} and the pelB leader sequence [25] which targets the apo-protein for translocation to the periplasm [26][3].

Since only Gram-negative expression systems were optimized to produce MHC, special attention had to be taken in heterologous expression of Gram-positive MHC using Gram-negative expression systems, such as *E.coli* and *S. oneidensis* MR1 strains. For instance, the *c*-Cyt maturation system often found in Gram-positive bacteria is system II or the Ccs system, instead of the Ccm found in Gram-negative bacteria. Unlike system I, system II possess four proteins that are responsible for the heme assembly, the CcsB, CcsA, ResA, and CcdA [4, 5]. In addition, Gram-positive bacteria use the Sec A [27–30] for protein translocation, instead of the SecB found in Gram-negative bacteria. This means that, the signal peptide sequence recognized by both Sec translocons are different, as well as the cleavage site for the respective signal peptidases. While in Gram-negative bacteria the signal peptidase cleavage occurs three to seven residues from the C-terminal end of the H-region (hydrophobic sequence of aminoacids that localizes between the N-terminal and the C-terminal), in Gram-positive bacteria this cleavage takes place preferentially from seven to nine residues from the same position [30, 31].

Due to the physiological differences stressed above, the heterologous overexpression of Gram-positive MHC is extremely challenging. However, it is of crucial importance for the purpose of this thesis which is the characterization of the proteins putatively involve in the EET of *T. potens* JR.

The functional characterization of the target proteins includes the elucidation of their redox properties and determination of their reduction potentials and how they are modulated by experimental conditions such as pH, temperature and interactions with physiological and non-physiological partners. The electrochemical and spectroscopic techniques described below were crucial to achieve this goal.

PROTEIN FILM VOLTAMETRY

Electrochemistry has been widely used as a tool to study interfacial and intramolecular electron transfer, substrate diffusion and protein interaction with small molecules, with extremely high temporal resolution and applied potential control [32]. This technique also enable the determination of the redox properties of redox-active proteins and the interfacial electron transfer between the proteins and insoluble electron acceptors, which in this case are the electrodes [33].

From all electrochemical methods, protein film voltammetry (PFV) is a simple and highly informative technique that allows to extract the protein reduction potential by varying the applied potential [34]. In this technique, the protein is directly adsorbed on the surface of the electrode, which facilitates the measurement of its reduction potential in different solution conditions, such as temperature, pH, buffers ionic strength to name a few [35, 36]. Since the film is ideally a monolayer of proteins, their active sites are easily accessible to the electrode and to the chemical

species present on the solution, such as protons, ions, ligands, or catalytic substrates. This feature allows the study of several reaction properties including electron transfer processes, conformational changes and even proton release often coupled with the electron transfer between the electrode and MHC proteins [37]. This technique requires very small amounts of biological material (1ul at a concentration of 30uM-50uM) to build the film on the electrode surface.

The major challenges of PFV are: i) the adsorption of the protein on the electrode surface ensuring that it is on its active configuration and ii) the interpretation of the voltammogram in a qualitative and quantitative level.

i) Adsorption of the protein on the electrode surface

Several electrode surfaces have been used to study proteins on their native conformation [38]. From these, pyrolytic graphite edge (PGE) seems to be the more successful in adsorbing native proteins [35, 36], due to their microscopically rough and chemically heterogeneous surfaces which allow proteins to attach to a suitable environment [39, 40]. Surface functionalization with natural based polymers like polymyxin [32], neomycin [32], chitosan[41] or poly-lysin [42] can also be used to promote the electrostatic interaction between the protein and the electrode, making the surface more suitable for protein adhesion.

ii) Interpretation of voltammetric results

The simplest voltammogram is produced by a one-electron redox center acquired at a scan rate low enough that the number of oxidized species is equal to the number of reduced species, as predicted by the Nernst equation [42, 43]. In this case, the peak separation is zero and both peaks

are symmetric with equal half-height widths. The peak height depends on the number of electrons, being more prominent as the number of cooperative electrons involved in the electron transfer process increases. By convention, the oxidation is represented by the positive current whereas the reduction assumes negative values [32, 43]. An illustrative voltammogram is presented on figure II.1 where both Faradic and capacitive current are depicted. The capacitive current is the sum of the current that flows through the electrode plus the current produced by both oxidation and reduction processes while the faradic current results from the subtraction of the electrode current from the current produced by the protein oxidation and reduction.

MHC are characterized by the presence of multiple redox centers which are interacting with each other [45]. PFV can successfully be used to addressed the reduction potentials of these proteins assuming that all centers or at least one can communicate with the electrode and intramolecular electron transfer is fast on the time scale of the experiment [32]. In this case, the reduction potential of individual redox centers cannot be determined being therefore macroscopic i.e. defined by the total number of electrons present in the system as a result of the contribution of all redox centers.

The discrimination of individual reduction potentials (microscopic states) in MHC is extremely difficult since the reduction potential of each redox center as well as the interactions between neighboring redox center must be considered [32]. Such interactions have different influence on each redox center, which translates into different shapes and positions of the titration curves. Flatter slopes are indicative of repulsive interactions (negative cooperativies) between the redox center whereas steeper slopes are produced by redox centers that are in positive cooperativity [45].

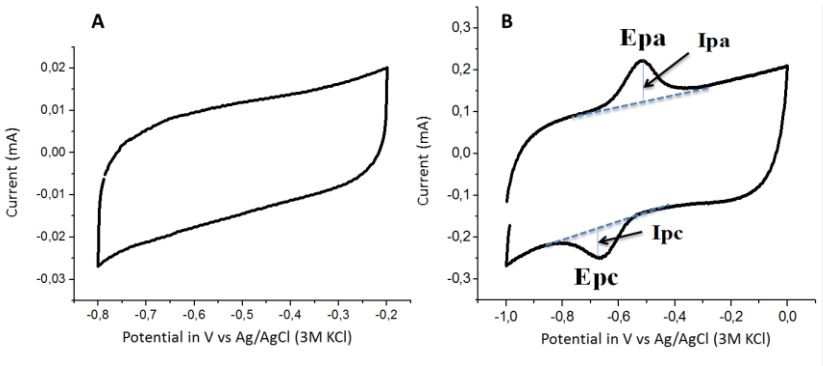


Figure II.1: Illustrative voltammogram A) capacitive current B) faradic current

Another parameter that must be taken into consideration is the Redox-Bohr effect. This heterotopic cooperative process arises from the thermodynamic coupling between redox centers and acid-base active centers which titrate at physiological pH [46, 47]. This proton-electron coupling is crucial for energy conservation [48, 49] and it is the major contributor to the cooperative interaction between redox centers. Thus, additional techniques that discriminate signals of individual redox centers like Nuclear Magnetic Resonance (NMR) or Electron Paramagnetic Resonance (EPR) can be used to assign individual reduction potentials to each heme.

NMR SPECTROSCOPY

Nuclear Magnetic Resonance Spectroscopy (NMR) is one of the most powerful techniques to study MHC. This spectroscopic technique is based on the magnetic momentum (nuclear spin) that some nuclei like ^{1}H, ^{13}C, ^{15}N or ^{31}P possess. When subjected to an external magnetic field the spin of these nuclei assumes a preferential orientation and the transitions between orientations occurs at different resonances frequencies that can be detected on the NMR experiment. The variation of the nuclear resonance frequencies due to variations in the electron distribution produces the chemical shift. Since the core of all proteins are mainly constituted by H, C and N, NMR can be used to study several functional and structural properties that characterize the proteins.

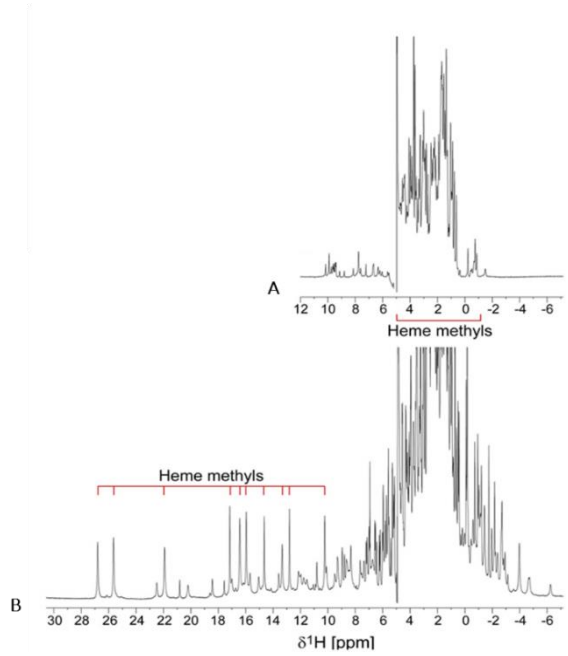


Figure II.2: NMR spectra of a MHC A) diamagnetic low spin state; B) paramagnetic low spin state

NMR is highly sensitive to the protein redox state which allows spectroscopic distinction of signals of the various hemes. In hemes with strong field ligands like histidines, the oxidized state is low spin paramagnetic (S=1/2) resulting on a shift of the methyl groups of the hemes to the low field region of the NMR spectra (0 – 40ppm) (Fig.II-

1B). This is a consequence of the paramagnetic effect caused by the unpaired electron from the iron on the chemical shift of the nuclei at the periphery of the hemes. In the reduced state the methyl signals remain in the diamagnetic region of the spectrum which corresponds to the protein envelope (Figure II.2A) [50, 51].

Due to this high sensitivity of the ^{1}H nuclei for the changes in their chemical environment, ^{1}H 1D- NMR can be used to predict the axial coordination of the iron of the hemes. In the case of Histidine-Methionine coordinated hemes, in the reduced state a peak at approximately -3ppm is indicative of iron coordinated methionine [52]. In the low spin paramagnetic the heme methyls are shifted up to 40ppm. On the other hand, high spin coordinated hemes (pentacoordinated) have their heme methyls shifted to spectral regions above 40ppm.

NMR can also be successfully used to study protein-protein [14, 18, 53–56] and protein-ligand interactions [21, 49, 50]. The interaction studies performed with this spectroscopic technique rely on the high sensitivity of the target nucleus, like ^{1}H, to changes in their chemical environment. To perform chemical shift perturbation experiments, samples containing a known concentration of target protein, in this case a cytochrome, are titrated by the addition of small volumes of concentrated solutions of another cytochrome (protein-protein interaction) or soluble electron shuttles (protein-ligand interaction). The changes in the peak position or line width of the target protein spectra will reveal the occurrence of interaction/binding. The chemical shift perturbation upon binding can be used to calculate the affinity constants between the interacting pairs [53].

To avoid spectral crowding in the ^{1}H frequency, other NMR active nuclei can be used to study protein binding. This is the case of ^{31}P 1D-NMR, where the signal produced by the inorganic phosphorous nucleus can be

used to investigate possible interaction between protein and molecules that contain phosphorous. This experiment is advantageous in the case of protein interaction with soluble molecules like FMN, which contains a single phosphorous nucleus [18, 21].

The study of protein interaction either with their physiological counterparts or environmental electron acceptors is of great importance to understand biological processes like electron transfer. In that regard, possible interactions must be measured to have an insight on the strength, duration and nature of the interaction which will allow to establish a correlation with the protein function. There are two main types of protein-protein and protein-ligand interaction, the weak/transient interactions and the strong/permanent interaction that form very stable complexes [59]. Dissociation constants ($Kd = \frac{koff}{kon}$, with k_{off} and k_{on} as dissociation and association rate constants, respectively) are used to calculate the strength of the interaction. These constant may cover a wide range of values that goes from micromolar (transient bindings) to nanomolar (permanent bindings) [60, 61]. On the NMR time scale, transient interaction is characterized by small chemical shift perturbation between free and bound states as a result of the changes on the chemical environment of the nuclei at the binding interface (fast-exchange regime). However, if the encounter complex does not dissociate, in the time scale of the experiment, a stable complex between protein and its ligand is formed. This specific complex has a greater chemical shift while compared with the same complex at a transient binding, since it has more population at the bound form. This often implies the visualization of only one of the forms which, during titrations, is

characterized by the reduction of peak intensity (slow-exchange regime) [62].

EPR SPECTROSCOPY

Electron paramagnetic resonance is the spectroscopic technique of excellence to give information about the paramagnetic centers by detecting characteristic magnetic features produced by the unpaired electron [63]. A single unpaired electron can exist in one of the two degenerate quantum electronic spin states +1/2 and -1/2 with the same energy. Under an applied magnetic field, the degeneracy is split as a function of the strength of the magnetic field. The energy separation between the spin states is given by $g\beta_e B_0$, where g is a proportionality factor similar to the chemical shift parameter of NMR, β_e is the electron Bohr magneton, and B_0 is the strength of the static magnetic field.

Typical EPR experiments are performed with a constant microwave frequency and a sweep magnetic field which induce spin-flip transitions. When the separation of the two spin states matches the constant microwave frequency the characteristic EPR derivative-shaped signal occurs [50, 64–66]. The g value depends on the orientation of the molecule with respect to the applied magnetic field and it can be defined as a tensor with principal values (g_x, g_y, g_z) arising from the magnetic axes of the paramagnetic center (x, y. z). In metal ions, the g tensor is determined by the nature and geometry of the axial ligands. While the free electron value g_e = 2.00232, in a molecular frame it can vary considerably [66]. If $g_x = g_y < g_z$ or $g_x = g_y > g_z$ the tensor is considered axial. In the case of hemes, the dihedral angle between the aromatic planes of axial histidine ligands are larger than 40º and the $g_z \geq 3.2$. On the other hand, if $g_x \neq g_y \neq g_z$ the tensor is rhombic with dihedral angles

for the aromatic planes of the axial histidine ligands of less than 40º and $g_z \leq 3.2$ [63, 66].

EPR experiments are performed at low temperatures that can range from 4K to 100K to slow down the rates of electron spin relaxation [67]. It is assumed that the structure and redox state of protein will not change significantly from that at room temperature [50, 66]. These experiments are performed with a concentrated protein solution (300µM minimum) at oxidized state.

The intensity of the EPR signals produced by the local environment of the unpaired electron is proportional to the number of paramagnetic centers. However, in the case of MHC the signals produced by the various redox centers often overlap making extremely complicated to distinguish individual centers. In that regard, redox titrations are performed to observe the behavior of the various redox centers upon changes on their redox state. These experiments are performed using a range of mediators that are chosen according to the reduction potencial range of the protein at a given pH. Figure II.3 depicts the redox mediators used to titrate the surface exposed protein at pH 7.6. Briefly, 20µM[66] - 80µM [68, 69] of each mediator are mixed with the concentrated protein solution and the desired reduction potentials are adjusted with concentrated solution of sodium dithionite. A small volume of the mixture (approximately 150 – 200µl) is removed from the bulk solution and transferred to the EPR tube that is immediately frozen on liquid nitrogen. All this procedure needs to be performed in anaerobic conditions to prevent changes on the previously establish reduction potential.

Due to the variation in the electronic structure caused by the coordination state and geometry of the iron in each heme the individual reduction

potential can be measured. Yet, the EPR signal of the various redox centers may still overlap which requires additional simulation techniques to deconvolute the individual contribution of each center [66].

FAST KINETIC TECHNIQUES

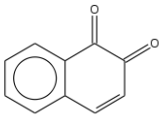
Most of biological intermolecular electron transfer processes occur on milliseconds time scale. To follow these processes *in vitro*, suitable techniques that can measure and follow such fast reactions must be used. The best way to measure the rate of chemical reactions is by mixing the reagent solutions in study and observe the changes. Yet, the speed of mixing and speed of observation are the limiting step. To overcome this situation, stopped-flow techniques were applied [70].

The stopped flow apparatus is a rapid mixing device that is used to study the kinetics of fast reactions in solution. This device consists of a sample handling unit that allows the rapid mixture of the samples and a UV-visible spectrophotometer to follow the changes in light absorption as function of time. The time between the end of solution mixing and the beginning of the observation of the reaction is called the dead time, and it is usually 1-3 milisseconds. The rapid monitorization of the reaction allows the use of small amounts of samples [71].

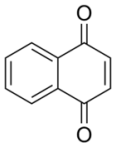
Due to the detection time scale of few milliseconds, this technique is suitable to study physiological reactions between interacting MHC [18, 55, 72], MHC and electron shuttles [21, 58, 73] as well as to study oxidation and reduction processes of MHC [19, 74–76]

The experiments with MHC are performed inside an anaerobic chamber to prevent the oxidation of the sample by the atmospheric oxygen. Both fully oxidized an reduced spectra of the protein are used as controls and are achieved by using and excess of potassium ferricyanide and sodium

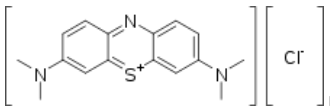
dithionite, respectively. To study the protein interaction with soluble electron shuttles (Figure II.4) small concentration of protein (less than 1μM) and an excess of electron shuttles (20μM) are used. The reaction is measured at a 552nm since at this wavelength the hemes display an intense change in absorption linked to the redox transition and the redox shuttles do not absorbed.



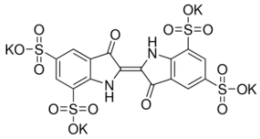
1,2-naphtoquinone (+180mV)



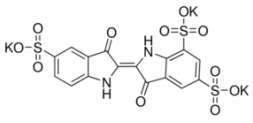
1,4-naphtoquinone (+60mV)



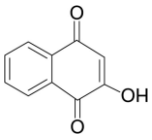
methylene blue (+11mV)



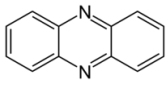
indigo tetrasulfonate (-70mV)



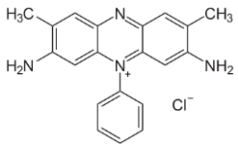
indigo trisulfonate (-30mV)



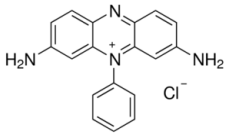
2-hydroxy-1,4-naphtoquinone (-152mV)



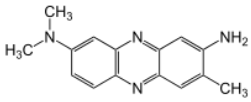
phenazyne (-125mV)



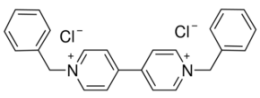
safranin (-280mV)



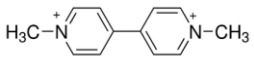
phenosafranin (-225



neutral red (-325mV)

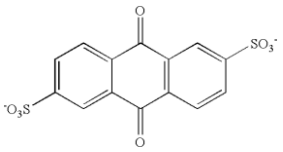


benzyl viologen (-345mV)

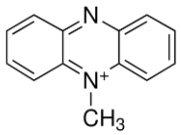


methyl viologen (-440mV)

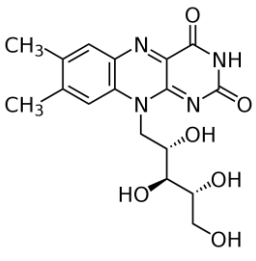
Figure II.3 – Redox mediators commonly used in EPR titrations at pH 7.6 [77]



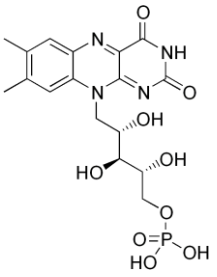
Antraquinone-2,6-dissulphonate (AQDS)



Phenazine methosulphate (PMS)



Riboflavin



Flavin mononucleotide (FMN)

Figure II.4: Commonly used redox electron shuttles

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Chapter III

Heterologous expression and purification of a multiheme cytochrome from a Gram-positive bacterium capable of performing extracellular respiration

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ABSTRACT

Microbial electrochemical technologies are emerging as environmentally friendly biotechnological processes. Recently, a thermophilic Gram-positive bacterium capable of electricity production in a microbial fuel cell was isolated. *Thermincola potens* JR contains several multiheme *c*-type cytochromes that were implicated in the process of electricity production. In order to understand the molecular basis by which Gram-positive bacteria perform extracellular electron transfer, the relevant proteins need to be characterized in detail. Towards this end, a chimeric gene containing the signal peptide from *Shewanella oneidensis* MR-1 small tetraheme cytochrome *c* (STC) and the gene sequence of the target protein TherJR_0333 was constructed. This manuscript reports the successful expression of this chimeric gene in the Gram-negative bacterium *Escherichia coli* and its subsequent purification and characterization. This methodology opens the possibility to study other multiheme cytochromes from Gram-positive bacteria, allowing the extracellular electron transfer mechanisms of this class of organisms to be unraveled.

KEY WORDS: Multiheme-cytochrome; Gram-positive bacteria; microbial fuel cells; *Thermincola*; extracellular electron transfer

INTRODUCTION

Microorganisms capable of performing extracellular electron transfer hold great potential for the development of environmentally friendly biotechnological applications. These are generally known as Microbial Electrochemical Technologies of which Microbial Fuel Cells (MFC) are the best-known example. These technologies have the potential to significantly improve the production of sustainable and renewable

energy, wastewater treatment processes, and implementation of sustainable biorefinery processes [1,2]. Both Gram-negative and Gram-positive bacteria are known to successfully transfer electrons directly to a solid anode in an operating MFC [3]. Studies performed on these devices showed that thermophiles produce higher levels of current than mesophiles in the same reactor, being often the prevalent electricity generating communities in the anode [4- 6]. Recently a thermophilic Gram-positive bacterium, *Thermincola potens* JR, was isolated in an operating MFC [7].

Studies exploring electron transfer in MFCs have taken place mainly on mesophilic Gram-negative bacteria like the gamma proteobacterium *Shewanella oneidensis* MR-1 and delta-proteobacterium *Geobacter sulfurreduces* PCA which electron transfer pathway are now well understood. In this organism multiheme *c*-type cytochromes (MHCs) transfer electrons from cytoplasmic and inner-membrane oxidizing enzymes towards redox super-complexes at the cell surface that are responsible for the reduction of solid phase electron acceptors [8]. When compared with Gram-negative bacteria, Gram-positive bacteria lack the outer membrane and present a thicker cell wall made of peptidoglycan. Moreover, the width of the periplasmic space between the membrane and the cell wall is smaller than the typical periplasm of Gram-negative bacteria [5,9,10]. These differences suggest a different electron transfer mechanism towards the cell surface. Interestingly, like the well-studied Gram-negative bacteria *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* [11], the genome of *T. potens* JR contains 32 genes that code for putative *c*-type cytochromes [12]. The physiological characterization of *T. potens* JR revealed that it is able to reduce insoluble ferric compounds [7]. Furthermore, a recent study by Carlson

and co-workers showed biochemical and biophysical evidence that MHCs are implicated in the reduction of insoluble ferric compounds [13]. However, to explore the mechanisms by which Gram-positive bacteria perform extracellular electron transfer it is necessary to characterize in detail the proteins involved in this process.

Escherichia coli is by far the best studied heterologous expression system for recombinant proteins [14,15]. However, the heterologous expression of *c*-type cytochromes requires additional care because it relies on specific cellular machinery to ensure the covalent binding of the heme to the apo-cytochrome. This process depends on the cytochrome *c* biogenesis system that ensures the translocation of the heme to the periplamic space and the covalent heme attachment to the apo-cytochrome [16,17]. The native cytochrome *c* maturation system, coded in *E. coli* by the gene cluster *ccm*ABCDEFGH [16-18] does not operate under aerobic conditions [19]. Therefore, co-expression of pEC86 system with the target *c*-type cytochrome is necessary for the proper maturation of the recombinant *c*-type cytochrome under aerobic conditions [20– 24].

In addition to the cytochrome *c* maturation system, the successful production of recombinant *c*-type cytochromes also depends on the translocation of the apo-protein to the periplasmic space where the heme assembly occurs [17,18]. The translocation to the periplasmic space is possible due to the presence of a specific signal peptide sequence. This signal sequence generally contains 15 to 30 residues and consists of three specific regions: the positively charged N-terminal region (N-region), the hydrophobic region (H-region) and the neutral C-region at the C-terminal end that is recognized by the signal peptidases in the periplasmic space [25,26]. Although for both Gram-positive and Gram-

negative bacteria the machinery of the Sec pathway responsible for the recognition of the hydrophobic N-terminal leader sequence and translocation of the apo-protein are very similar, the cleavage of this sequence by peptidases is quite distinct [25,27]. While in Gram-negative bacteria the signal peptidase cleavage occurs three to seven residues from the C-terminal end of H-region, in Gram-positive bacteria this cleavage takes place preferentially from seven to nine residues from the same position. Therefore, special care has to be taken in the heterologous expression of Gram-positive *c*-type cytochromes in Gram-negative bacteria like *E. coli*.

In this paper, we present, for the first time, the heterologous expression and purification of a multiheme *c*-type cytochrome from a Gram-positive bacterium. In order to ensure the proper cleavage of the target protein, the signal peptide of the periplasmic decaheme protein ThrJR_0333 from *T. potens* JR was replaced with the signal peptide from the small tetraheme cytochrome *c* from *S. oneidensis* MR-1 (SO_2727), an abundant constitutively expressed protein [28–30]. This approach enabled the over-expression of the Gram-positive decaheme cytochrome TherJR_0333 in the Gram-negative bacterium *E. coli.* This methodology will facilitate studies aimed at elucidating the mechanisms of extracellular electron transfer performed by Gram-positive bacteria that colonize anodes, and will contribute to the understanding and improvement of microbial electrochemical technologies such as MFCs.

MATERIALS AND METHODS

I.1 Protein Identification

- **Bacterial strain and growth conditions**

The sequence of the signal peptide derived from the small tetraheme cytochrome *c* (STC) from *S. oneidensis* MR-1 was used to produce a chimeric gene with *therJR_0333* gene from *T. potens* [29], using the primers listed in table 1 (NZYtech, Portugal). The *stcsp*_FW primer was designed according to Shi *et al.* [31] to clone efficiently the chimeric gene (named *stcsp_therJR0333*) into pBAD202/D-TOPO vector (Invitrogen, USA). Cloning was performed according to manufacturer specifications, and the final expression vector (pCP01) was inserted into the *E. coli* strain JM109 (DE3) co-transformed with pEC86, which contains the *ccm*ABCDEFGH genes [32]. Positive transformants were used for expression tests, where different media, induction times and inducer concentrations were tested. Bug-buster reagent (Novagen, USA) was used to check the best over-expression condition. The selected condition was later used to over-express TherJR_0333. Briefly, the transformants were grown in Terrific Broth (TB) with 50 $\mu g.ml^{-1}$ kanamycin and 34 $\mu g.ml^{-1}$ chloramphenicol in 5 liter Erlenmeyer flasks containing 2 liters of medium and 1% of inoculum at 37°C and 150 rpm. At mid-log phase (about 6 h of growth) protein expression was induced with 1 mM of arabinose, the temperature was lowered to 30°C and cells were allowed to grow for additional 16h. Cells were harvested by centrifugation at 10 000g for 10min at 4°C.

Table III.1: Oligonucleotides used to construct the chimeric gene

Primers	Sequence
stcsp_FW	CACCTAAGAAGGAGATATACATCCCGTGAGCAAAAAAC TATTAAG
therJR0333_RV	TTATTTTTTCTGTGCCTGCTCTGCAGCCGG
stc_therJR0333_FW	CAACCGCATTTGCCACTGCTCCCGAGAAG
stc_therJR0333_RV	CTTCTCGGGAGCAGTGGCAAATGCGGTTG

- **Protein purification**

The cell pellet was suspended in an osmotic shock solution (0.5 M sucrose, 0.2 M Tris-HCl, 0.5 mM EDTA and 100mg.L^{-1} lysozyme, pH 7.6) containing protease inhibitor cocktail (Sigma) and DNase I (Sigma) [33]. This mixture was incubated at 4ºC for 30min with gentle stirring. The supernatant containing the periplasmic fraction was cleared by ultracentrifugation at 20 000g for 1h at 4ºC, dialyzed overnight against 20 mM Tris-HCl (pH7.6) and concentrated in an ultrafiltration cell with a 10 kDa cut-off membrane. The resulting sample was loaded onto an ion exchange diethylaminoethyl (DEAE) column (GE Healthcare) previously equilibrated with 10 mM Tris-HCl (pH 7.6). The resulting fractions were eluted with a salt gradient from 0 to 1 M KCl in the same buffer. The chromatographic fractions were followed by UV-visible spectroscopy and SDS-page (12%) to select those containing the protein of interest. The target protein, TherJR_0333, was eluted at 100mM Tris-HCl with 100mM NaCl. The fractions containing TherJR_0333 were concentrated and analyzed by UV-visible spectroscopy and SDS-PAGE (12% gel) to check for the presence of further contaminating proteins. Over time, the fraction containing TherJR_0333 protein started to form

a red precipitate. Solubilization of this precipitate was achieved using 10 mM potassium phosphate buffer (pH7.6) with 100 mM potassium chloride and 0.05% of the non-ionic detergent n-Dodecyl-β-maltopyranoside (DDM). Since the addition of the detergent prevented further precipitation of the target protein, the latter characterization was performed with solubilized protein using 0.05% DDM. A single band on the SDS-Page gel confirmed the purity of the target protein, and the purity index of the sample was defined by $A_{Soret\ peak}/A_{280nm}$ ratio.

- **Mass spectrometry**

Protein intact mass was assessed by MALDI-TOF/TOF. The data were acquired in positive linear MS mode using a 4800plus MALDI-TOF/TOF (AB Sciex) mass spectrometer and using 4000 Series Explorer Software v.3.5.3 (Applied Biosystems). External calibration was performed using Promix3 (Laser BioLabs).

II.1 Spectroscopic characterization

- **UV-visible spectroscopy**

UV-visible spectra of solubilized TherJR_0333 in 10mM potassium phosphate buffer (pH 7.6) with 100 mM KCl and 0.05% DDM were acquire on a Shimadzu UV-1800 spectrophotometer in the range of 250-800nm at room temperature. Reduced spectra were obtained by adding an excess of sodium dithionite (Sigma) to the oxidized sample. Protein concentration was estimated using the absorption coefficient of $\varepsilon_{409} = 125\ 000\ M^{-1}\ cm^{-1}$ per heme for the oxidized state of the protein.

- **NMR spectroscopy**

10% (v/v) of D_2O was added to the protein sample prepared solubilized in 10mM potassium phosphate buffer (pH 7.6) with 100mM KCl and 0.05%DDM before spectral acquisition. NMR experiments were performed on a Bruker AVANCE II 500MHz spectrometer equipped with a TXI probe. The ^{1}H-NMR spectrum was acquired at 25^{o}C with spectral width of 80 kHz, and processed in the Topspin 3.2 software from Bruker using an exponential apodization function and a line broadening of 40.

RESULTS AND DISCUSSION

Multiheme *c*-type cytochromes are major components of electron transfer networks in metal reducing bacteria, being essential for the extracellular electron transfer processes performed by these organisms [3,8,13]. The decaheme cytochrome TherJR_0333 from the Gram-positive bacterium *T. potens* JR was identified as a participant in extracellular electron transfer in this bacterium [13], perhaps playing a similar role to the periplasmic decaheme cytochrome MtrA from *S. oneidensis* MR-1 [34]. The heterologous expression of this protein was successfully achieved in the Gram-negative *E. coli*.

The production of *c*-type cytochromes requires specific cell machinery, such as the cytochrome *c* maturation systems and Sec translocation system that are generally different between Gram-negative and Gram-positive bacteria. Protein BLAST (NCBI NIH) reveals that both *E. coli* and *T. potens* JR have the same cytochrome *c* maturation system, which is the Ccm system I. However, while *E. coli* uses Sec B as the major translocation system, *T. potens* JR uses Sec A [35]. Thus, to ensure that the target protein TherJR_0333 will be recognized by the *E. coli* SecB

translocation system the signal peptide of STC (SO_2727) was used [29,28]. Synthetic oligonucleotides were designed to contain the signal peptide of STC from *S. oneidensis* MR-1, that is known to be constitutively expressed in high levels [29,36]. Although the use of the STC signal peptide was previously shown to be highly efficient for over-expression of *c*-type cytochromes [28,29], this is the first time that it was used to over-express a multiheme *c*-type cytochrome from a Gram-positive bacterium in a Gram-negative bacterium.

Protein expression was assessed on SDS-page followed by heme-staining coloration to confirm the protein-bound heme. Figure II.1 depicts the result of protein expression tests of *E. coli* JM109 (DE3) co-transformed with pEC86, TherJR_0333 with native signal peptide (lanes 2 and 3) and TherJR_0333 with STC signal peptide (lanes 4 and 5) respectively. The appearance of a band around 40 kDa, estimated to be the molecular weight of TherJR_0333 with 10 hemes [13] was only observed when the native signal peptide of TherJR_0333 was replaced by the STC signal peptide.

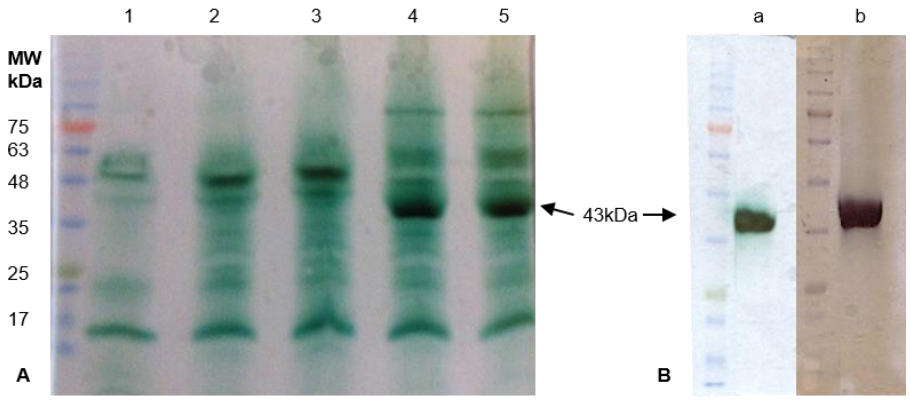

Figure III.1: 12% SDS-Page of TherJR_0333. A) Expression tests using TB medium: Lane 1 – *E. coli* JM109 with pEC86; Lane 2 – *E. coli* JM109 with pEC86 and JR0333_pBAD; Lane 3 - *E. coli* JM109 with pEC86 and JR0333_pBAD with 1mM arabinose; Lane 4 - *E. coli* JM109 with pEC86 and pCP01; Lane 5 - *E. coli* JM109 with pEC86 and pCP01 with 1mM arabinose B) Solubilized precipitate: Lane a – heme staining; Lane b – Comassie blue R. Arrows indicates the expressed TherJR_0333.

Figure III.2: Diagram of heterologous protein containing the STC signal peptide and TherJR_0333 sequences. The cleavage site AFA-TA was confirmed by N-terminal analysis.

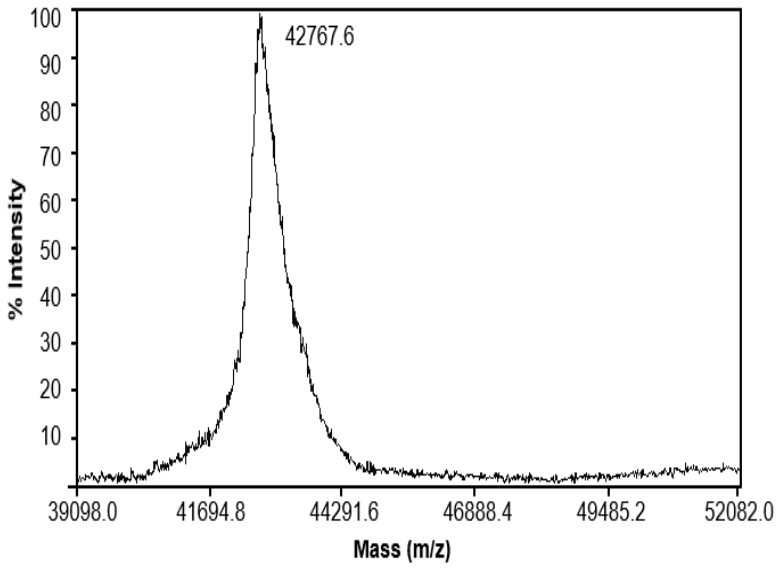

Figure III.3: MALDI-TOF/MS spectrum of recombinant TherJR_0333

Pure protein was obtained by solubilization of the red precipitate that was formed in the fraction eluted with 10mM Tris-HCl using 10 mM potassium phosphate buffer and 100 mM KCl. To prevent protein precipitation 0.05% of DDM was added to the solubilized solution. Since the periplasmic space of Gram-positive bacteria is narrower than that of Gram-negative bacteria and crossed by several membrane bound polymers [10] it is likely that the detergent mimics the native environment by creating micelles that stabilize the target protein. In fact, little precipitate formation was observed in the sample after the addition of DDM. Solubilized recombinant protein gave a single heme-stained band on SDS-PAGE gel between the 35kDa and 48kDa markers (Figure III-1B_lane a). Coomassie blue confirmed the purity of sample by revealing the absence of any other protein (Figure 1B_lane b).

N-terminal sequencing (Figure III) confirms that Sec B from *E. coli* recognizes the signal peptide of STC allowing the biogenesis of the

Gram-positive *c*-type cytochrome TherJR_0333 in the periplasmic space of a heterologous Gram-negative expression system. Indeed, the expression of TherJR_0333 using the native signal peptide (in JR0333_pBAD vector) did not yield the production of *c*-type cytochrome (Figure 1A _lanes 2 and 3). This result clearly indicates that the presence of a recognizable signal peptide is essential for the heterologous expression of proteins when using identical translocation systems. MALDI-TOF mass spectrometry analysis of the solubilized protein revealed a molecular mass of 42 767.6 Da (Figure III.3). This value agrees with the calculated molecular mass of 42.7 kDa considering the incorporation of the 10 hemes and a molecular mass of 616.5 Da per each heme.

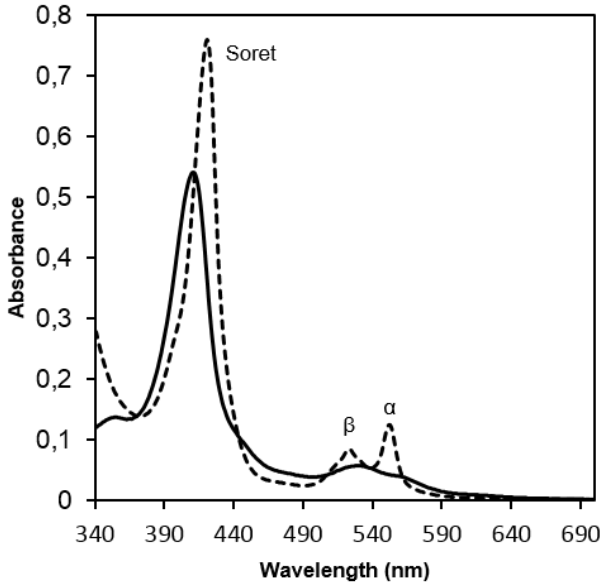

Figure III.4: Reduced (dashed line) and oxidized (solid line) UV-visible spectra of TherJR_0333. The sample concentration was 100µM in 10mM potassium phosphate buffer (pH7.6) with 100mM KCl and 0.05% DDM.

The UV-visible spectra of solubilized recombinant TherJR_0333 are shown in Figure III.4. This protein exhibits the typical UV-visible spectrum of a low spin hexacoordinated *c*-type cytochrome with absorption peaks at 409nm (Soret peak) and 530nm in oxidized form (solid line) and 416nm (Soret), 525nm (β-band) and 552nm (α-band) in the reduced state (dashed lines) [37]. These spectral changes confirm that the solubilized TherJR_0333 can undergo oxidation/reduction processes. The pure solubilized protein displays a ratio of the Soret peak in the oxidized state with the absorbance at 280nm of 3.

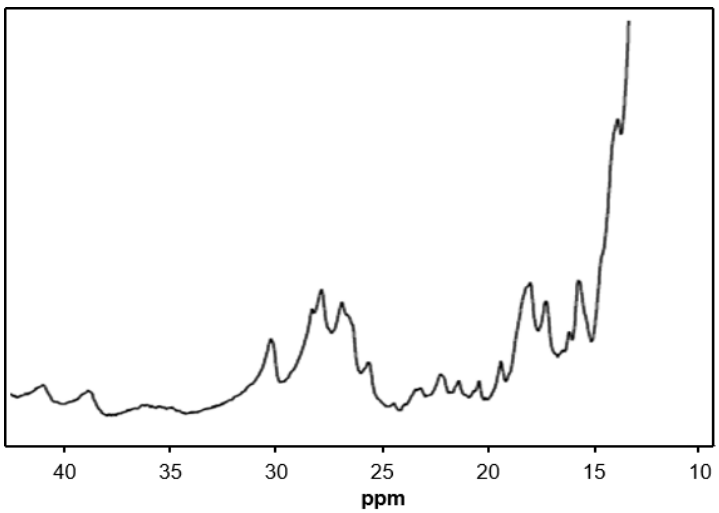

Figure III.5: ^{1}H-NMR spectrum of TherJR_0333 at 25ºC in the oxidized form in 10mM phosphate buffer (pH7.6) with 100mM KCl and 0.05% DDM at 25ºC.

The NMR spectrum of solubilized recombinant protein TherJR_0333 shown in Figure III.5 exhibits the typical NMR signals of a low spin *c*-type cytochrome, where peaks from the methyl groups of the hemes are shifted to the paramagnetic field are seen from 15ppm to 40ppm [38,39]. The polypeptide sequence of TherJR_0333 reveals ten canonical heme binding motifs CXXCH and nine spare histidine residues, which means

that not all the ten hemes are bis-histidine coordinated. Since the aminoacid sequence of TherJR_0333 also shows the presence of three methionine, NMR experiments with reduced sample were performed showing the characteristic fingerprint for His-Met coordinated hemes with a peak around –3 ppm that corresponds to the ε-CH3 protons of the iron coordinated methionine [40]. The NMR spectrum obtained for the sample purified by precipitation is identical to the spectrum obtained for the soluble fraction (data not shown).

CONCLUSION

Previously, only monoheme *c*-type cytochromes from Gram-positive bacteria were heterologously expressed in a Gram-negative expression system using specific plasmids that contain the pelB leader sequence [41,42]. In the present study, a different approach was used, where the signal peptide of a constitutively expressed periplasmic multiheme *c*-type cytochrome from *S. oneidensis* MR-1, STC, was added to the N-terminus of the decaheme periplasmic protein TherJR_0333 from the Gram-positive *T. potens* JR. Using this approach, we were able to over-express a multiheme cytochrome from a Gram-positive bacterium in a Gram-negative bacterium for the first time. This construct is also highly versatile since it allows the over-expression of the heterologous protein in both *E. coli* and *S. oneidensis* MR-1 [31]. This methodology creates the opportunity to study other *c*-type cytochromes from Gram-positive bacteria, paving the way to study electron transport conduits across bacterial cell walls of Gram-positive bacteria.

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Chapter IV

Gram positive bacteria do it differently? Characterization of the cell surface multiheme cytochrome OcwA from *Thermincola potens* JR

This chapter is part of a published article:

Costa, N.L., Hermann, B., Fourmond V., Faustino, M., Teixeira, M., Einsle, O., Paquete,C.M., Louro, R.O, *How thermophilic Gram-positive organisms perform extracellular electron transfer: characterization of the cell surface terminal reductase OcwA,* mBio **DOI: 10.1101/641308**

ABSTRACT

Extracellular electron transfer is the key biochemical process that allows the development of microbial electrochemical technologies (MET). Despite the differences in the cell envelope, Gram-positive bacteria were found to perform direct extracellular electron transfer to solid electron acceptors using multiheme *c*-type cytochromes. This discovery was a breakthrough in the field of MET.

In this work, the functional and structural characterization of OcwA, a multiheme surface exposed protein from the Gram-positive bacterium *Thermincola potens* JR, is described. Fast kinetic techniques using stopped flow showed that OcwA can reduce insoluble ferric electron acceptors and is also able to react with soluble redox shuttles, including flavins. In contrast with the surface exposed cytochromes from Gram-negative bacteria, OcwA shows hemes with a more varied coordination including Hi-His, His-Met and hemes without a distal aminoacid ligand bound to the iron. The structural characterization revealed that OcwA also has a different and more packed architecture of the heme core from that observed for Gram-negative outer-membrane cytochromes. These differences might be related with the higher current production by *T. potens* JR in a MFC while compared with mesophilic electroactive species like *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* PCA.

Overall, the data obtained in this work points to an adaptation of the multiheme cytochromes as key players in extracellular electron transfer in these phylogenetically distant organisms. Moreover, this work is pioneer on the exploration of the role of multiheme cytochromes in

extracellular electron transfer on Gram-positive bacteria, broadening the view of this important process for the development of MET.

KEY WORDS: multiheme cytochromes; surface exposed proteins; Gram-positive multiheme proteins;

INTRODUCTION

The discovery that Gram-positive bacteria can perform extracellular electron transfer (EET) was a breakthrough in the field of Microbial Electrochemical Technologies (MET). Unlike Gram-negative bacteria, Gram-positive bacteria are enveloped by a thicker cell wall (30 to 100 nm), lack an outer membrane, and contain a narrow periplasmic space (10nm approximately) which is filled with low-density material [1–4]. This type of structural arrangement was for several years considered incompatible with direct extracellular electron transfer (EET) to insoluble electron acceptors. However, the Gram-positive bacterium *Thermincola potens* JR was isolated from the anode of a MFC inoculated with anaerobic sludge that was operating at 55ºC [5].

Physiological characterization of *T. potens* JR showed that it couples acetate oxidation with reduction of insoluble hydrous ferric oxides (HFO) and antraquinone-2,6-disulfonate (AQDS). Interestingly, this bacterium failed to reduce soluble iron derivatives like ferric citrate suggesting an obligate utilization of insoluble ferric electron acceptors [6]. MFC studies showed the presence of a biofilm at the anode supporting a direct contact mode for electron transfer and cyclic voltammetry analysis of bacterial growing medium showed no presence of small soluble redox shuttles [5]. Such features were previously observed on *Geobacter* spp. where filtrates of the culture medium failed to stimulate the reduction of Fe(III) over the rate that was observed in

fresh medium or upon addition of 10µM of AQDS [7,8] supporting a preferential direct contact for insoluble ferric oxides rather than production of redox shuttles [9].

Electricity generation studies performed on MFC inoculated with anaerobic sludge revealed that *T. potens* JR was responsible for 70% of the produced electrical current [6]. Furthermore, pure culture studies showed that *T. potens* JR was able to generate more current (0.42mA) than the representative electrogenic *S.oneidensis* MR1 (0.03 – 0.3mA) [10] and *G. sulfurreducens* PCA (0.25mA) [11] in the same type of reactor [5, 6].

Genome sequencing of *T. potens* JR revealed the presence of 32 genes that code for putative multiheme *c*-type cytochromes (MHC) [12]. As observed for the representative electroactive Gram-negative bacteria *Shewanella oneidensis* MR1 and *Geobacter sulfurreducens* PCA, MHC are directly linked to a heme-based strategy for EET towards the reduction of insoluble electron acceptors [13, 14], such as Fe and Mn minerals [15, 16] or electrodes in METs [10, 11, 17, 18].

Biochemical and biophysical characterization of *T. potens* JR lead to the identification of four MHC that are putatively involved in the reduction of insoluble hydrous ferric oxide and in direct contact with the surface of electrodes, these are: TherJR_1117, presumed to be attached to the membrane, the TherJR_1122, proposed to be non-covalently associated with the cell wall polymers, TherJR_0333, proposed to be periplasmic and TherJR_2595 that is known to be exposed on the surface of the cell. These results lead to the proposal of a hypothetical EET pathway for this bacterium [19]. Interestingly, unlike its Gram-negative counterparts TherJR_2595 is not embed on a porin like structure but proposed to be bound to the surface of the cell wall [19].

Due to their localization at the interface between bacteria cell surface and the extracellular environment the outer membrane cytochromes (OMC) from the representative electroactive bacteria play a crucial role on both direct and indirect contact with the insoluble ferric substrates [20–27]. Thus, to understand the distinct performance of Gram-negative and Gram-positive bacteria in MFCs it is of great interest to unravel the functional mechanisms of this class of proteins in both microbial types.

In this paper, we describe the functional and structural characterization of the first cell-surface exposed multiheme *c*-Cyt from a Gram-positive bacterium, TherJR_2595 here after referred as OcwA (Outer cell wall protein A), with a putative role on EET at bacteria-electrode interface. OcwA is a 59kDa nineheme cytochrome found to be exposed on the cell surface of *T. potens* JR when the bacterium is grown anaerobically with acetate as electron donor and hydrous ferric oxides (HFO) or antraquinone-2,6-disulfonate (AQDS) as electron acceptors [19].

With this work, the authors hope to contribute with additional knowledge on the surface exposed proteins that can be found in electroactive microorganisms. The main goal is to broaden the spectrum of novel microorganisms that can be used on the anode of METs systems with highlight on this thermophilic electroactive bacterium which holds a great potential for energy production and wastewater treatment [28, 29].

MATERIALS AND METHODS

Protein Identification

- **Expression and purification**

The OcwA protein was expressed according to Costa *et al.* 2015 [30] with minor changes. Briefly, a chimeric gene that contains the signal peptide from *Shewanella oneidensis* MR-1 small tetraheme cytochrome

(STC) and the gene sequence of the target protein OcwA lacking its native signal peptide was constructed using the primers listed in table V.1. The chimeric gene was cloned into pBAD202/D-TOPO vector that contains a V5 epitope and a His-tag at the C- terminus of the expressed protein according to the literature [31]. *E. coli* JM109 (DE3) co-transformed with plasmid pEC86, which contains the *ccmABCDEFGH* genes for expression of *c*-type cytochromes in aerobic conditions [32] was used to overexpress the recombinant protein OcwA. Cells were grown at 37°C in TB medium containing 50μg/ml of kanamycin and 35μg/ml of chloramphenicol. At mid log phase, approximately 6h after inoculation, the temperature was lowered to 30ºC and the cells were allowed to grow for additional 18h. The pBAD202/D-TOPO vector has a *ara*BAD promoter which is positively regulated by the *ara*C gene. Expressions tests performed with arabinose induction, with concentrations ranging from 0 to 2mM, revealed that no significant increase of heterologous protein was achieved upon induction.

The cells were pelleted by centrifugation at 10.000xg for 10min at 4ºC and resuspended in osmotic shock solution (0.5M sucrose, 0.2M Tris-HCL, 0.5mM EDTA, 100mg/L lysozyme, pH7.6) with protease inhibitor (Sigma) and DNase (Sigma). The spheroplast solution was incubated at 4ºC for 40min with gentle stirring. The periplasmic fraction containing the recombinant protein was cleared by ultracentrifugation at 200.000xg for 1h at 4ºC and dialyzed overnight against 20mM Tris-HCl (pH 7.6).

The dialyzed protein extract was purified in a His-trap column (GE Helthcare) previously equilibrated with 20mM sodium phosphate buffer, 20mM Imidazole and 500mM NaCl (pH 7.6). To elute the bound protein a 20mM sodium phosphate buffer with 300mM Imidazole and 500mM

NaCl (pH 7.6) was used. The resulting fraction was dialyzed overnight against 20mM Tris-HCl (pH 7.6) to remove the imidazole.

A diethylaminoethyl (DEAE)-biogel column (Biorad) was used as a final purification step. Briefly, the column was equilibrated with 20mM Tris-HCl (pH 7.6), and 20mM Tris-HCl buffer with 1M NaCl (pH 7.6) was used to elute the proteins. OcwA was eluted with 20mM Tris-HCl buffer with 120mM NaCl (pH 7.6) and concentrated OcwA was dialyzed overnight against 20mM potassium phosphate buffer (pH 7.6) with 100mM KCl.

SDS-PAGE (12% gel) and UV-visible spectroscopy were used after each purification step to select the fractions containing the target protein and to evaluate its purity.

Table IV-1: Oligonucleotides used to construct the chimeric gene

Primers	Sequence
Forward_STC_pBAD	**CACCTAAGAAGGAGATATACATCCC**GTGAGCAAAAAACTATTAAG
Reverse_2595_pBAD	TTATTGGAGTTTCTGTTCCGCAGCGGTTAACAGCTTAACG
Forward_STC_2595	**CCAACCGCATTTGCC**GAAAAGCCTGCGGACA
Reverse_STC_2595	TGTCCGCAGGCTTTTC**GGCAAATGCGGTTGG**

- **Mass spectrometry**

Protein intact mass was assessed by MALDI-TOF/TOF. The data were acquired in positive linear MS mode using a 4800plus MALDI-TOF/TOF (AB Sciex) mass spectrometer and using 4000 Series Explorer Software v.3.5.3 (Applied Biosystems). External calibration was performed using Promix3 (Laser BioLabs).

Crystallization and structure determination

Crystals were grown in an anoxic tent under N2/H2 atmosphere at room temperature. Sitting drop vapor diffusion experiments were set up by adding 0.5 µL of protein solution (6 mg/mL) to 0.5 µL of reservoir solution. Good diffracting crystals were obtained in conditions with 24-27% PEG 3350, 0.1 M HEPES, pH 7-7.5 and 0.1-0.2 M $MgCl_2$ only after seeding.

Crystals were harvested using 10% 2-, 3-butanediol in mother liquor and flash frozen in liquid nitrogen. Datasets were collected at the Swiss lights source, Villigen, Switzerland at beamline X06DA with a Pilatus 2M Detector.

Initial phases were obtained by single anomalous diffraction with a single dataset measured at the iron edge of 1.729 Å using the PHENIX suite [33] for automatic phasing and initial model building. A first structure was refined in iterative steps using REFMAC5 [34] and COOT at 2.7 Å resolution. This solution was then used as search model for molecular replacement with MOLREP [35] as part of the ccp4 suite [36] for the higher resolved structure at 2.2 Å.

Spectroscopic Analysis

- **^{1}H NMR**

Oxidized OcwA prepared in 20mM potassium phosphate buffer (pH 7.6) with 100mM KCl were lyophilized and resuspended in 500µl of D_2O to a final concentration of 300µM. Sodium dithionite was used to reduce the protein. NMR experiments were performed on a Bruker Avance II 500MHz spectrometer equipped with a QXI probe for ^{1}H detection. The ^{1}H NMR spectra were acquired at 25ºC with a 100kHz spectral window

and processed in the Topspin 3.2 software from Bruker using an exponential apodization function with LB=40Hz. ^{1}H NMR spectra were acquired before and after lyophilization to ensure that protein integrity was preserved.

- **^{31}P-NMR binding**

To observe the interaction between FMN and OcwA samples containing 100µM of FMN were prepared and titrated against increasing concentrations of OcwA. ^{31}P-1D-NMR spectra were recorded for each addition at 55ºC on a Bruker Avance II 500MHz spectrometer equipped with a ^{31}P detection SEX probe. The pH of the samples was measured before and after each series of additions to confirm that the pH of the solution remained unchanged. Chemical shift perturbations were measured according to Diaz-Moreno et al. 2005 [38]

- **EPR**

OcwA protein solution was prepared in 20mM potassium phosphate buffer (pH 7.6) with 100mM KCl to a final concentration of 200 µM. Fully oxidized spectrum was performed prior to the titration. Fully reduced spectrum was achieved using borohydride. The redox titration was performed in an anaerobic cell flushed with argon. A cocktail of mediators (80µM each 1,2-naphtoquinone, 1,4-naphtoquinone, methylene blue, indigo tetrasulphonate, indigo dissulphonate, 2-hydroxy-1,4-naphtoquinone, phenosafranine, safranine, neutral red, benzyl viologen, methyl viologen) and phenazine (>50µM) was added to accelerate the redox equilibrium. Glucose oxidase (280nM), catalase (640nM) and glucose (1mM) were also added to redox titration mix to ensure the consumption of any free O_2. Reduction steps were performed by adding small amounts of 40mM sodium-dithionite (pH 9). After each

reduction step, 200µl of sample was withdrawn from the redox titration mix and frozen in liquid nitrogen (77K).

The reduction potentials were measured with a combined platinum Ag/AgCl electrode calibrated at 25 °C. All potentials are quoted against the standard hydrogen electrode. EPR spectra were collected on a Bruker ESP 380 spectrometer equipped with an ESR 900 continuous-flow helium cryostat from Oxford Instruments.

Biochemical characterization

- **Protein Film Voltammetry**

The electrochemical setup was assembled as previously described [33][34]. The electrochemical experiments were performed in an anaerobic glovebox (JACOMEX, France) filled with nitrogen (residual O_2 < 1ppm). A pyrolytic graphite edge electrode (PGE surface approx. 3mm^2) was polished with alumina slurry (Buehler, 1µm) then coated with 0.5µl of a solution of OcwA (stock solution previously diluted in 20mM potassium phosphate buffer (pH 7.6) with 100mM KCl to a final concentration of 50µM) and left to dry for approximately 5min.

The buffers used in the electrochemical experiments were prepared by mixing 5mM of HEPES, MES and TAPS and 100mM KCl. The desired pH values were adjusted with 1M NaOH or HCl solutions.

Experiments were performed at 25ºC using an electrochemical cell consisting of an Ag/AgCl (saturated KCl) reference electrode in a Luggin sidearm and a platinum wire counter electrode. Cyclic voltammetry was performed with an Autolab electrochemical analyzer (PGSTAT-128N) with an analogue scan generator controlled by GPES software. The potentials were quoted with reference to standard hydrogen electrode (SHE) by addition of 0.197 V to those measured [41].

The electrochemical data were analyzed using an open source program available at www.qsoas.org [42].

- **Oxidation experiments with redox shuttles**

The protein oxidation experiments were performed according to Paquete *et. al* [43] in a stopped-flow apparatus (SHU-61VX2 from TgK Scientific) placed inside an anaerobic chamber (M-Braun 150) containing less than 5ppm of oxygen. Briefly, dilutions of the OcwA protein to desired concentrations were made from a degassed stock solution of OcwA in 20mM potassium phosphate buffer with 100mM KCl (pH7.6). The exact concentration of protein was determined by UV-visible spectroscopy using ε_{409nm} of 125.000 M^{-1} cm^{-1} per heme for the oxidized state of the protein [38, 39].

5mM stock solutions of the electron shuttles antraquinone-2,6-disulfonate (AQDS), flavin mononucleotide (FMN), riboflavin (RF) and phenazine methosulfate (PMS) were prepared by dissolving weighted amounts of solid reagents in 20mM potassium phosphate buffer (pH 7.6) with 100mM KCl. Dilutions of the electron shuttles were prepared in degassed buffer, and their concentrations were measured by UV-visible spectroscopy using the following extinction coefficients ε326nm = 5200 $M^{-1}cm^{-1}$ for AQDS [46], ε_{445nm} = 12,200$M^{-1}cm^{-1}$ for FMN[47], ε_{445nm} = 12,500 $M^{-1}cm^{-1}$ for RF [48], and ε_{387nm} = 26,300$M^{-1}cm^{-1}$ for PMS [49]. Reduced OcwA was obtained by mixing the protein with small volumes of concentrated sodium dithionite solution. UV-visible spectroscopy was used to confirm that there was no excess of dithionite using ε_{314nm} =8.000 M^{-1} cm^{-1} [50].

Oxidation by electron shuttles was monitored by measuring the light absorption changes at 552nm upon mixing reduced OcwA protein with

each of the electron shuttles. The temperature of the kinetic experiments was maintained at 289K ±1 using an external circulating bath [43].

Calculation of rates of Intramolecular electron transfer

The rate of room temperature intramolecular electron transfer was estimated using the Mosser-Dutton ruler [51] considering a first order approximation:

$$\text{Log } k = 13 - 0{,}6\ (R - 3.6) - 3.1\ (\Delta G + \lambda)^2/\lambda$$

where k is the rate in units of s^{-1}, R is the edge-to-edge distance in Å, ΔG is the driving force for electron transfer between donor and acceptor in eV and λ is the reorganization energy in eV, taken to be approximately 1eV [52, 53].

RESULTS AND DISCUSSION

To characterize OcwA, heterologous expression and purification of the recombinant protein was performed according to previous methodology establish in our group for the characterization of the periplasmic protein TherJR_0333 [30]. The expression of OcwA was confirmed by SDS-Page that revealed the presence of a band near 60kDa after heme staining treatment [54]. The purity of the expressed protein was revealed by incubation of the SDS-Page gel with Blue Safe solution (Nzytech) which did not show the presence of any further proteins (Figure VI.1A). Mass spectrometry confirm that the purified protein has a molecular weight of 59kDa which corresponds to the predicted mass of the polypeptide sequence of OcwA plus the nine heme insertion (Figure VI.1B). Spectroscopic characterization showed that pure protein had the typical UV-vis spectrum of a low spin *c*-type cytochrome at both oxidized and reduced state with an absorbance ratio ($A_{\text{Soret peak}}/A_{280\text{nm}}$) larger than 5.0

(Figure IV.2). The yield of OcwA was 1.5mg per liter. The 1D ^{1}H NMR spectrum of OcwA in the oxidized state showed signals up to 40 ppm, that are typical of low spin heme methyls (Figure IV.3), while the 1D ^{1}H NMR spectrum of the reduced protein revealed the presence of a peak around -3ppm. This corresponds to the ε-CH3 group of iron coordinated methionine characteristic for His-Met coordinated hemes (S.I Figure VI.1) [55].

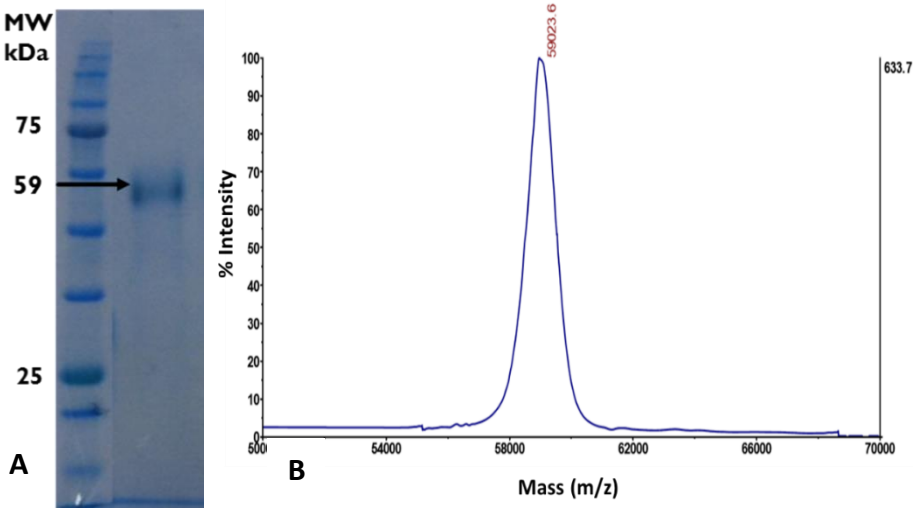


Figure IV.1: A) 12% SDS–Page of OcwA with both heme staining and Blue safe.; B) MALDI-TOF/MS spectrum of recombinant OcwA

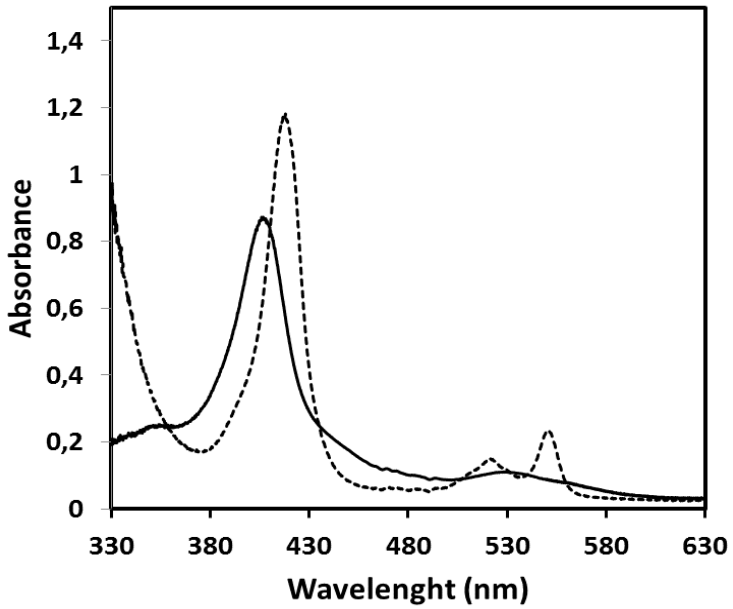


Figure IV.2: UV-visible spectra of OcwA in the reduced (dashed) and oxidized (solid line) form

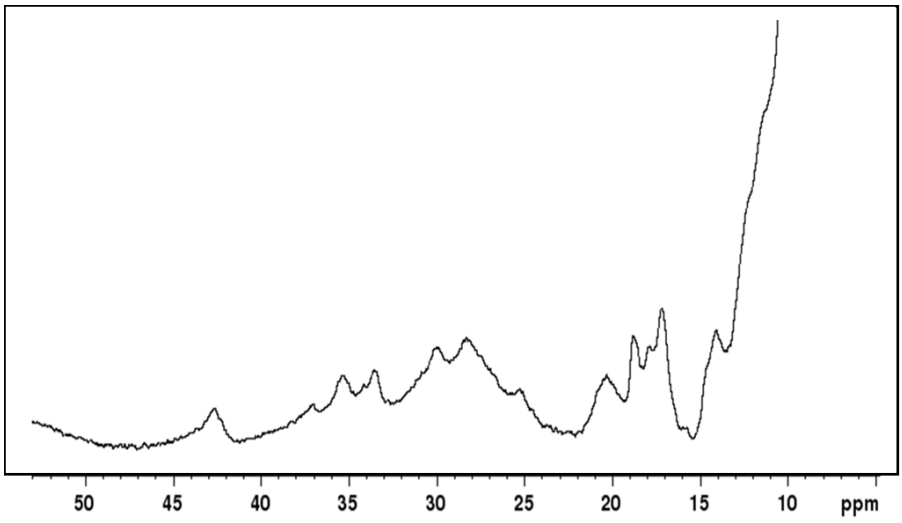


Figure IV.3: ^{1}H-NMR spectrum of OcwA at 25^{o}C in the oxidized form

The X-ray structure of OcwA was solved at 2.2Å resolution (S.I Table VI.1). The structure revealed 3 types of heme coordination: bis-histidine coordinated hemes (I, III, IV, VI, VII, VIII); one histidine-methionine coordinated heme (IX); and two hemes with a histidine coordination position in the proximal side and an open coordination on the distal side (II and V) (Figure VI.4). The difference between the dihedral angles planes of the imidazoles in the coordination of the low spin hemes of OcwA was measured from the structure (Table IV.2).

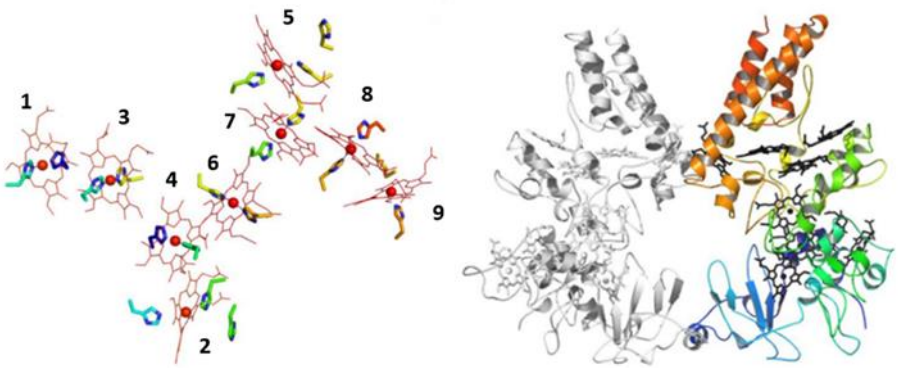


Figure IV.4: Preliminary structure of OcwA at 2.2Å.

Table IV-2: Diffrence between the dihedral angles planes of the imidazoles in the coordination of the low spin hemes of OcwA.

Hemes	Dihedral angles	Axial ligand coordination type
I	10º	rhombic
III	20º	rhombic
IV	43º	axial
VI	21º	rhombic
VII	50º	axial
VIII	60º	axial
IX	35º	-

The EPR spectrum of the OcwA also revealed the presence of three intense g-values that correspond to at least three types of heme coordinations (Figure IV.4). While the peak at g~6 indicates the presence of high-spin hemes, the peaks at g=3.5 and g=2.8 are characteristic of low spin hemes with different geometry of the axial ligands. From structure analyses and the results from the table VI.3, it could be seen that the hemes IV, VII and VIII have the imidazole ring planes of the His-His axial ligands in a axial disposition which corresponds to the intense peak of g~3.5. The hemes I, III and VI have rhombic His-His with the ring planes nearly occluded and thus contributing to the peak with g~2.8 [56]. Considering that hemes II and V have open coordination on the distal side they are the most likely to give rise to the high spin signal in the EPR spectrum with g=6.

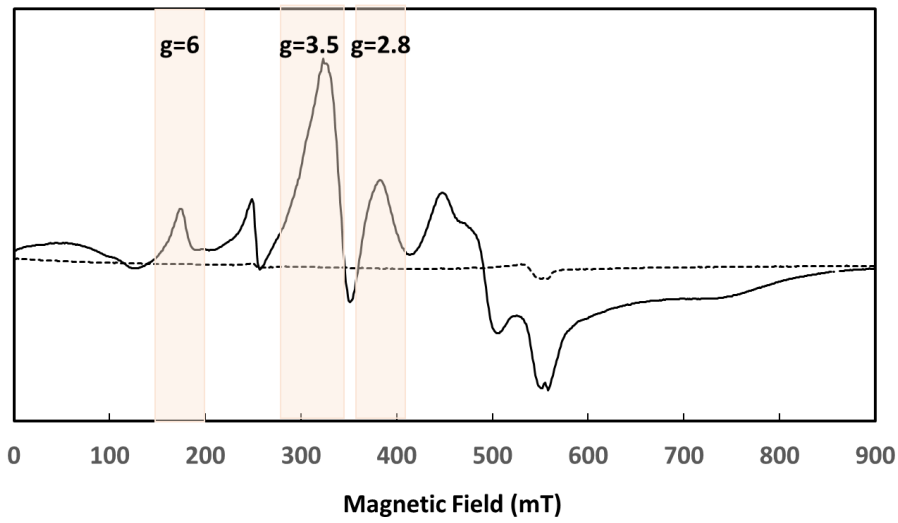


Figure IV.5: X-band EPR spectra of OcwA at 7K: oxidized protein (solid line), fully reduced (dashed line) with borohydride.

Surface exposed MHC are key players in both direct and indirect EET to insoluble electron acceptors. In direct EET the immediate contact between bacteria and the external environment is ensured either by the cell surface attached proteins or cellular appendages like pili or nanowires [57]. In indirect EET, the contact between the surface exposed *c*-Cyts and the insoluble electron acceptors is mediated by soluble electron shuttles that are produced either by the bacteria or exogenously available in the growing media [58, 59]. To study the redox behavior of OcwA in direct contact with insoluble electron acceptors, protein film voltammetry (PFV) was performed. This technique is highly suited to study surface exposed redox proteins [59, 60] like OcwA allowing an insight of its reduction potential upon direct contact with insoluble electron acceptors, such as the anode of a MFC. PFV showed that pure OcwA forms a stable film at the surface of the PGE electrode demonstrating reversible electrochemistry over a wide potential window ranging from +100mV (fully oxidized) to -400mV (fully reduced) vs SHE (Figure IV.6). This reduction potential window is similar to that previously observed for the outer membrane multiheme *c*-Cyts (OMCs) from *S. oneidensis* MR-1 and *G. sulfurreducens* that are known to participate in the reduction of insoluble terminal electron acceptors [61, 62]. Furthermore, this broad range of potentials covers the reduction potential of AQDS, which is known to be reduced by *T. potens* JR [19], and also the reduction potential of soluble electron shuttles such as Flavin mononucleotide (FMN), Riboflavin (RF) and Phenazine methosulfate (PMS) (Table VI.3) documented to facilitate the reduction of insoluble compounds on MFC [43, 64–68].

Table IV-3: Commonly used electron shuttles

Electron shuttles	E° (mV)	Reference
AQDS	-185	
FMN	-216	[69]
Riboflavin	-206	
PMS	+80	[70]

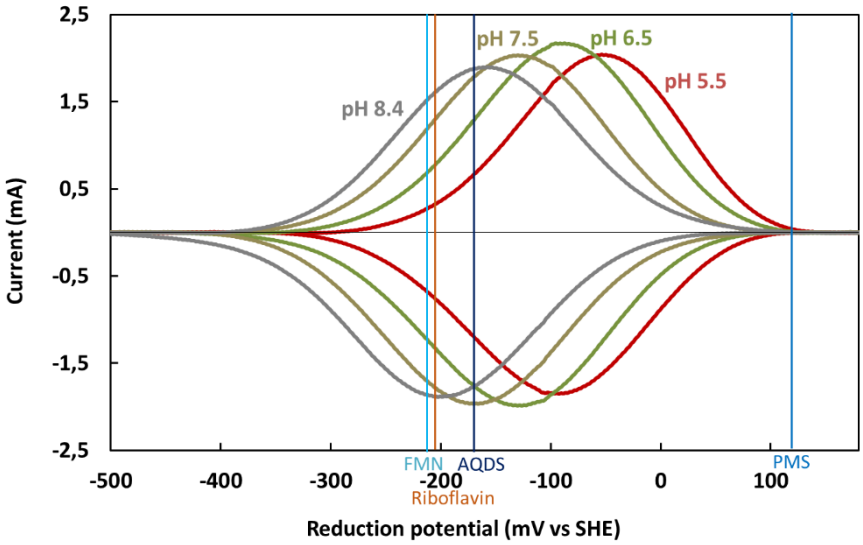


Figure IV.6: Cyclic voltammetry of OcwA obtained by PFV at 25ºC at 200 mV/s

The ability of bacteria that do not produce endogenous electron shuttles, but are able to use them when present in the external environment to enhance the EET process has already been revealed in *Geobacter* spp. [7, 71]. To investigate if *T. potens* JR is able to interact with soluble electron shuttles when they are present in the environment, the oxidation of the OcwA by AQDS, FMN, RF and PMS was measured by stopped flow (Figure IV. 7). The experiments were performed with an excess of

electron shuttles, and as expected from their intrinsic reduction potentials (Table IV. 3) only PMS was able to completely oxidize OcwA. The incomplete oxidation of OcwA by electron shuttles other than PMS is indicative of a thermodynamic equilibrium between the protein and the electron shuttles. These results show that, OcwA, can reduce environmentally relevant soluble electron shuttles in addition to direct reduction of the insoluble electron acceptors. Reduction of iron minerals and soluble compounds was also observed for other Gram-positive organisms such as *Carboxydothermus ferrireducens* [72] and *T. ferriacetica* [73–75] and in both cases MHC were proven to be involved in the process. Interestingly, a similar pattern for reduction of electron shuttles was previously observed for the OMC from *S. oneidensis* MR1 [43] supporting the reduction potential window obtained in this study for OcwA.

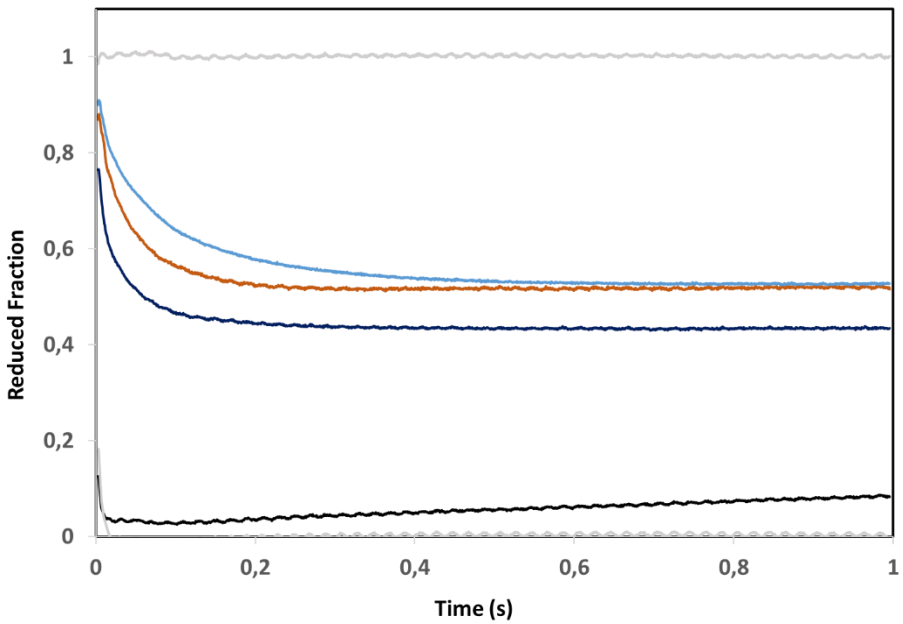


Figure IV.7: Oxidation of OcwA by FMN (light blue), Riboflavin (orange), AQDS (blue) and PMS (black).

From the three-dimensional structure of OcwA, the loose coordination at the distal side and high solvent exposure, makes hemes II and V good candidates for the sites of interaction with soluble extracellular redox active compounds.

To understand the molecular bases of the interaction between OcwA and FMN, ^{31}P-1D NMR spectra were performed by following the phosphorous signal of FMN upon titration with increasing amounts of protein. Since no other phosphorous atom exists in the protein or in the FMN, the NMR spectra will only depict the signals of FMN and the phosphate buffer producing a less crowded spectra. The results showed that increasing amounts of OcwA do not lead to significant changes on the chemical shifts of the FMN signal but instead there was a decrease in peak intensity as well as an overall broadening of the signal (Figure IV.8). These peak changes demonstrate that the ligand binding to OcwA occurs in the slow exchange regime in the NMR time scale which is indicative of a strong binding. Yet, this ligand binding behavior is different from what was previously reported for the OMC from *Shewanella oneidensis* MR-1, OmcA and MtrC, that establish transient bindings with flavins [43]. This feature could also be related with the selective advantage of *T.potens* JR over other bacteria from the thermophilic MFC community from which it was identified as the prevalent species [6].

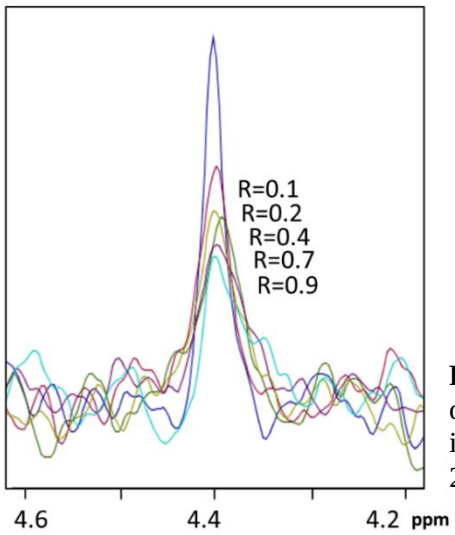


Figure IV.8: ^{31}P-1D NMR spectra of FMN in the presence of increasing amounts of OcwA at 25ºC

Currently OMC from *Shewanella spp.* are the only surface exposed *c*-Cyts structures available for comparison with OcwA. Those structures presents ten [76–78] or eleven [79] hemes covalently bound forming a staggered cross structure with 6 hemes arranged along the length of the protein and the remaining four perpendicularly positioned in relation to the other six. At the edges of the tetraheme chain two extended Greek key split β-barrel domains can be found and are likely to be the FMN binding site [80]. Plus, for all the OMC characterized structurally up to now, all hemes are low spin His-His coordinated and tightly packed (edge to edge distances between 4Å and 6Å) which ensures a rapid inter-heme electron transfer [81, 82].

As can be seen for the OcwA structure, the internal heme packing in this protein is novel and does not fall into the typical staggered-cross arrangement from the OMC. Instead the architecture of the heme core align with the octaheme sulfite reductase MccA from *Wollinela succinogenes* [82] and the pentaheme nitrate reductase NrfA [83] two

proteins actively involved in electron transfer process towards sulfite and nitrite reduction (Figure IV.9).

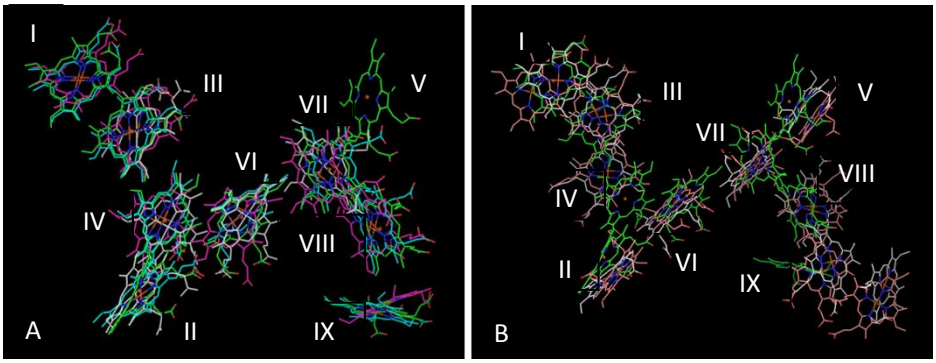


Figure IV.9: Structural alignment of the OcwA hemes (green) with A) MccA (blue), HAO (pink) and NrfA (grey) and B) MtrC (salmon) and OmcA (light pink)

Table IV- 4: Edge-to-edge distances between the hemes.

Hemes	C - C (Å)	$Log_{10}Ket$
I - III	4,1	10
II - IV	3,6	10
III - IV	6,4	8
IV - VI	4,5	9
V - VII	4	10
VI - VII	6,6	8
VII - VIII	5,6	9
VIII - IX	5,8	9

The edge-to-edge distances between the hemes were calculated (Table IV.5) and was found that 6 hemes are in a "stacked" arrangement with each other exhibiting the smallest distances (3.6 – 4.5Å) and the remaining 3 hemes are in the T-shaped pairing (5.6 – 6.6Å). No coplanar

motifs were found in this structure between neighboring hemes which makes the overall distances between the 9 hemes systematically shorter than those presented by the previously characterized OMCs. Using the Moser-Dutton ruler [51] the intramolecular electron transfer rates were estimated. Figure IV.10 depicts the schematic representation of the intramolecular pathway in OcwA. As can be seen for the color scheme the fastest electron transfer pathway occurs over the pentaheme chain from hemes 2⇔5 in both ways. Hemes I and II seem to branch from heme IV as well as hemes VIII and IX which seems to branch from heme VII. As said previously hemes II and V are the most likely to be entering and exiting points due to their high solvent exposure. Since the structure does not give a clear hint on how the protein is positioned on the cell wall one cannot be sure of the entering and exiting points. However, considering the perfect alignment with the hemes of the sulfite reductases one can speculate that heme II is possibly the entering point for the electron. The relatively conserved heme orientation on these three structures places OcwA heme II in the same position as MccA heme VIII and NrfA heme V which is on close contact with the cell wall. Furthermore, OcwA heme V aligns perfectly with the active site of the sulfite reductases hemes II and I of MccA and NrfA respectively (Figure VI.9) [82, 84].

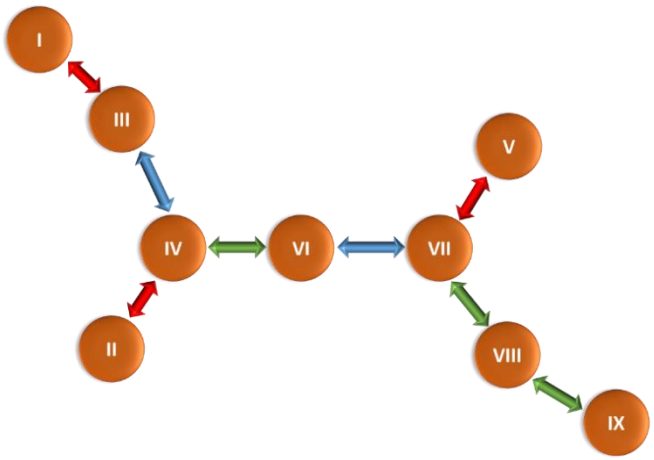


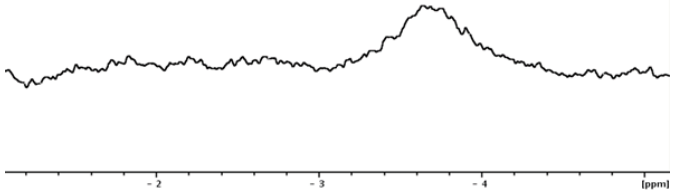
Figure IV.10: Intramolecular electron transfer pathway of OcwA. Red arrows k_{et}=10; green arrows k_{et}=9 and blue arrows k_{et}=8

CONCLUSIONS

This study reposts the comprehensive characterization of the cell surface exposed protein from *T. potens* JR, OcwA. This is the first functional and structural report of an electrode reducing redox protein displayed at the surface of the cell wall of a Gram-positive bacterium. OcwA reveals a blend of features that are common to previously characterized OMC from Gram-negative bacteria with others that are unique and broaden our perception of the breadth of solutions evolved by nature to perform extracellular electron transfer. These findings raise several questions regarding the evolution of the surface exposed proteins involved in EET of different electroactive microorganisms. For example, what is the reason for OcwA to have a structure similar to proteins involved in the reduction of sulfites despite being a terminal reductase like the OMCs? Why is the OcwA heme arrangement and coordination so distinct from the OMCs? Do these differences relate to possible physiological advantage in both environment and MFCs?

Taken together, the results from this study are pioneer and opens new perspectives on the multitude of proteins that might be involved in EET processes shading light on the structural differences that might confer the same biological function.

SUPPORTING INFORMATION



S.I Figure IV-1 : Reduced 1D ^{1}H-NMR spectrum of OcwA at reduced state showing the characteristic peak of the methyl of the heme coordinated methionine.

S.**I Table IV-1**: Data collection and Refinement Statistics.

Data Set	**OcwA – SAD**	**OcwA – high res**
space group	*P1*	*P1*
wavelenght [Å]	1.728963	1.00000
cell constants *a*, *b*, *c* [Å]	55.48, 63.16, 84.53	54.85, 62.97, 84.21
α, β, γ [°]	101.81, 99.38, 98.24	101.54, 99.13, 98.40
resolution limits [Å]	49.61-2.70 (2.83-2.70)	49.06– 2.20 (2.27 – 2.20)
completeness (%)	89.9 (90.2)	98.1 (97.3)
unique reflections	26,907 (3,591)	53,356 (4,603)
multiplicity (%)	3.7 (3.8)	3.5 (3.4)
R_{merge} [a]	0.071 (0.35)	0.075 (1.29)
$R_{p.i.m.}$	0.043 (0.21)	0.047 (0.83)
mean I/ σ(I)	11.7 (3.5)	11.0 (1.0)

Cc (1/2)	0.996 (0.895)	0.998 (0.4)
refinement statistics		
R_{cryst} [b]		20.2
R_{free} (%)		23.3
non-hydrogen atoms		8280
solvent molecules		1
Cruickshank's DPI [85]		0.31
r.m.s. deviations from ideal values		
bond lengths (Å)		0.010
bond angles (º)		1.416
protein main chain atoms		59.40
protein all atoms		60.44
solvent		79.99
Wilson plot		58.67
Residues in most favored regions		97.3 % (937/963)
In additional allowed regions		99.8% (961/963)
In generously allowed region		

[a] $R_{merge} = \Sigma_{hkl} [(\Sigma_i |I_i - \langle I \rangle|) / \Sigma_i I_i]$

[b] $R_{cryst} = \Sigma_{hkl} ||F_{obs}| - |F_{calc}|| / \Sigma_{hkl} |F_{obs}|$

R_{free} is the cross-validation R value for a test set of 5 % of unique reflections

[c] Ramachandran statistics as defined by Molprobity [86]

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Chapter V

Concluding Remarks and Future Perspectives

GENERAL DISCUSSION AND CONCLUDING REMARKS

This thesis describes for the first time the development of a methodology to overexpress and produce MHC from Gram-positive bacteria using Gram-negative bacteria such as *E. coli* and *S. oneidensis* MR1 as expression system. Briefly, a chimeric gene containing the signal peptide of the small tetraheme cytochrome *c* from *S. oneidensis* MR-1 was fused to the truncated gene *therjr_0333* (without native signal peptides) which encodes for the periplasmic decaheme TherJR_0333. This construct was cloned into pBADTOPO/D-202 vector and co-expressed with pEC86 resulting in high yields of recombinant periplasmic MHC.

The purification of this protein was quite challenging since after the first elution step on DEAE, with 10mM Tris-HCl using 10 mM potassium phosphate buffer and 100 mM KCl, a red precipitate was formed indicating low protein solubility and stability. To overcome this situation, micellar detergents such as n-Dodecyl β-D-maltoside (DDM) and lately sodium-cholate were added. The need to stabilize the protein using detergents is a strong indicative of crowded Gram-positive periplasmic environment ruled by different protein charges and interactions. The addition of Na-cholate to the protein solution turned out to ensure more soluble protein yield than DDM. The reason for this might be related with the chemical nature of the detergents, i.e. while DDM is nonionic, Na-cholate is anionic[1, 2]. Plus, the micelles produced by DDM are larger than the ones produced by Na-cholate [1, 3] which means that Na-cholate is less prone to form protein clusters while compared with DDM. 1D ^{1}H -NMR spectroscopy experiments with TherJR_0333 without detergents and with both detergents, DDM and NA-cholate respectively were performed. Na-cholate spectrum is the

one that resembles the most the spectrum of TherJR_0333 without detergents, thus the one that mimics better the protein folding obtained after purification (S.I Figure V.1). Furthermore, considering that the periplasmic space of Gram-positive bacteria is very narrow and is filled with distinct types of proteins, the effects of the surface charge might be more intense on these proteins [4]. Thus, by having a negative charge, the micelles produced by Na-cholate might mimic better the native environment of TherJR_0333.

The biochemical characterization of the decaheme protein TherJR_0333 was achieved through spectroscopic techniques such as UV-vis spectroscopy and NMR that allowed to conclude that a fully functional MHC that can undergo oxidation and reduction was produced. Furthermore, NMR enabled to evaluate the coordination of the hemes, showing that upon reduction of the protein a characteristic peak of the εCH3 from methionine appeared around -3ppm indicating the presence of a His-Met coordinated heme. 1D ^{1}H-NMR experiments with large sweep width (200ppm) performed in both 300MHz and 500MHz did not show any signal beyond 45ppm indicating that no high spin coordinated hemes are present in this structure (Figure V.2). Considering that the polypeptide sequence of TherJR_0333 has 19 Histidines, it is plausible say that the remaining 9 hemes are low spin His-His coordinated (S.I Figure V.2).

Without a native protein to compare, the correct spectrum of TherJR_0333 thus the correct protein form is still unknown. Yet, the results obtained from this work clearly show the expression and purification of a fully functional MHC.

The methodology developed for the heterologous expression of the periplasmic decaheme from *T. potens* JR, was used to successfully

produce high yields of the recombinant nineheme OcwA. This allowed a thorough functional and structural characterization of the first surface exposed MHC from a Gram-positive bacterium.

The purification of OcwA was less problematic since the protein kept soluble throughout subsequent purification steps without the need of detergents. Enzymatic treatment of intact bacterial cells using trypsin demonstrate that OcwA have a high trypsin/lysed ratio which is a great indicative not only of its subcellular localization at cell surface but also of the lack of tight bound to the peptidoglycan cell wall. This might be the reason why the protein was more soluble than TherJR_0333, which in the same study were shown to have inferior trypsin/lysed ratio than OcwA [5].

Using PFV it was demonstrated that OcwA has a wide range of reduction potential that expands from +50mV to -450mV depending on the pH used. This proton-electron coupled mechanism it's a way to ensure the packaging of several redox centers into a single protein without an increase in repulsive (anti-cooperative) effects caused by the electrons in the hemes. Furthermore, it allows operating MFC using only a single metabolic source that can either provide electrons for the establishment of an electrical current as well as protons that will cross the proton exchange membrane where they will be combined with other electrons and O_2 to form water and thus closing the circuit at the cathode. This feature is transversal to all the MHC that participate in EET pathways characterized so far and it's the major advantage of their usage on microbial electrochemical technologies.

The wide reduction potential window of OcwA was also shown to cover the reduction potential of insoluble ferric electron acceptors and AQDS

as well as the reduction potential of the commonly used soluble redox shuttles such as FMN, Riboflavin and PMS known to interact with outer-membrane cytochromes (OMCs) from *S. oneidensis* MR-1and *G.sulfurreducens*. Stopped flow experiments revealed that like the OMCs OcwA can also reduce these electron shuttles. Yet, unlike the OMCs, binding affinity experiments followed by 1D ^{31}P-NMR showed unequivocally that OcwA binds strongly to FMN. The functional consequences of such binding require further studies.

Structural characterization of OcwA revealed three types of heme coordination including Hi-His, His-Met and hemes without a distal aminoacid ligand bound to the iron. This structural characterization obtained by X-ray crystallography revealed that OcwA has a different heme architecture from that observed for Gram-negative outer-membrane cytochromes (i.e. staggered cross arrangement). Instead, OcwA presents an heme core arrangement that is highly similar to the one observed for reductases of nitrogen compounds, the octaheme cytochrome MccA, the nitrate reductase NrfA and hydroxylamine oxidoreductase (HAO). This was the most curious result of this study and probably the one that rises more fundamental question regarding the evolution of the proteins involved in the EET and opens more room for further studies on putative OcwA-like proteins.

To date, the way that OcwA is bound to the cell wall remains unclear, however, the solved structure might give some hints about the possible binding site and type of chemical bounding. Considering that the peptidoglycan is negatively charged, due to the presence of embedded teichoic acids, the proteins on its surface must be attached by a positive charged site of the polypeptide chain [6]. Using pymol, positive charged residues (Lysin and Arginine) and negative charged residues (Glutamic

acid and Aspartic acid) were selected and marked along all OcwA polypeptide chain (S.I Figure V.3). By doing so, it was possible to observe that both positive and negative charges are almost equally distributed along the OcwA surface, yet a cluster of positive charge residues can be found on the loop around heme II. In addition, the environment surrounding the heme II has higher prevalence of polar residues. Altogether, the net charge around heme II is suitable for the protein attachment to the cell wall since the positively charged residues will be attracted by the negative charged peptidoglycan and the polar residues will enable the protein attachment by hydrogen bounds. The predicted intramolecular electron transfer showed that the fastest pathway occurs over the pentaheme chain from hemes 2⇔5 in both ways. Homology structure prediction (Swiss model) showed that the structure of NrfA is the one that fits better the structure of OcwA with a sequence identity of 23.68%, followed by HAO, cytochrome *c* nitrite reductase (ccNir) and lastly the MccA. Thus, considering the perfect alignment between the solved structure of OcwA and the proteins mentioned above, and the identity between this protein and NrfA one can speculate that like NfrA the surface around OcwA heme II is the protein binding site to the cell wall. This makes the heme II a plausible candidate to be the entering point for the electrons that result from the bacterial metabolism.

The structural and functional characterization of OcwA was carried out at 25ºC. This temperature is far from the physiological temperature that *T. potens* JR grows, however, NMR spectra acquired at both 25ºC and 55ºC showed no further differences besides peak shifting and spliting of some signals as a result of temperature increase (S.I). Plus, increasing in

the temperature will not change the protein thermodynamic instead it will only affect the kinetic of the reactions, which will be accelerated.

Overall, it is fair to say that the methodology developed in this thesis was crucial for its continuity and it shed light on the comprehensive knowledge of the first characterized Gram-positive MHCs. Due to its versatility, this methodology can also serve as a standard procedure for future recombinant protein production using heterologous expression systems like *E.coli* and *S. oneidensis* MR1.

In short, the study presented on this thesis was pioneer and opened a new door for further investigations on distinct characteristics that might be presented on cell surface exposed proteins of already identifiedelectroactive microorganism. Furthermore, it paves the way for fundamental studies that aim to understand the role played by Gram-positive proteins in EET processes.

There is still a long way to go so that electroactive microorganisms can be used in devices that make the most of their potential and with this work I hope to have contributed with a few more bricks for the construction of this "yellow brick road".

FUTURE PERSPECTIVES

Significant efforts have been performed towards the understanding of the molecular mechanisms by which Gram-positive *T.potens* JR perform EET, however, there is much more to do to prove the hypothetical EET pathway proposed by Carlson and its co-workers [5].

Production of the remaining MHC, the membrane bound TherJR_1117 and the cell wall imbedded TherJR_1122, proposed to be involved in *T potens* JR EET is of crucial importance to in vitro reconstitution of this hypothetical pathway.

A functional and structural characterization of TherJR_0333 needs to be carried out to understand how this protein interacts with its proposed physiological partner the membrane bound TherJR_1117. Investigate if it also interacts with the periplasmic hydrogenases TherJR_0764-0765 that are alternatively proposed to initiate the EET process, as well as with the cell wall imbedded TherJR_1122. This later protein is not homologous to any characterized MHC and is one of the most prevalent proteins in the *T. potens* JR cell extracts indicating an abundancy in the electron transfer pathway [5].

The arrangement of TherJR_1122 in the hypothetical pathway seems to indicate that this protein establishes a wire of self-repeated units arrangement along the cell wall which may constitute an interesting mechanism used by other Gram-positive dissimilatory metal reducing bacteria to ensure the electron transfer across the packed cell wall. In addition, the hypothetical EET model prosed for *T. potens* JR gives the idea of a putative exposure of this protein to the extracellular environment, so, it would be interesting to test the electron transfer between TherJR_1122 and insoluble terminal acceptors to see if it is an alternative pathway for the EET. The scientific relevance of this protein is high, and its characterization will certainly be a major contribution to understand the mechanisms by which Gram-positive bacteria inserts this MHC in the peptidoglycan cell wall.

Furthermore, overexpression of TherJR_1122 will allow to see if in fact the latter and OcwA transfer electrons to each other or if there are any other physiological partners for OcwA that are yet to be identified.

Overall, these future studies will enable the establishment of a general mechanism for EET transfer in electroactive Gram-positive bacteria

namely *T. ferriacetica* which shares 99% identity with *T. potens* thus sheding light on putative EET pathways on other Gram-positive bacteria.

With regards to biotechnological applicability, the benefits of operating electrochemical devices at thermophilic conditions are immense and includes: (i) thermal and chemical stability of the bioreactors with drastic reduction on the solubility of O_2 which enables more effective and large-scale fermentation processes; (ii) high microbial metabolism due to the higher physiological temperatures reduces the associated costs on temperature control for heating or cooling during industrial-scale fermentations; (iv) high bioconversion of carbon substrates like biopolymers, hydrocarbons, and aromatics compounds with industrial added value; (v) thermal pathogenic inactivation.

High temperature MFCs can be used for treatment of several sources of wastewaters that ranges from alcohol distilleries until municipal waters with a high cost-effective manner since the energy cost of the remediation of the polluted streams will be compensated by the produced energy throughout the industrial process.

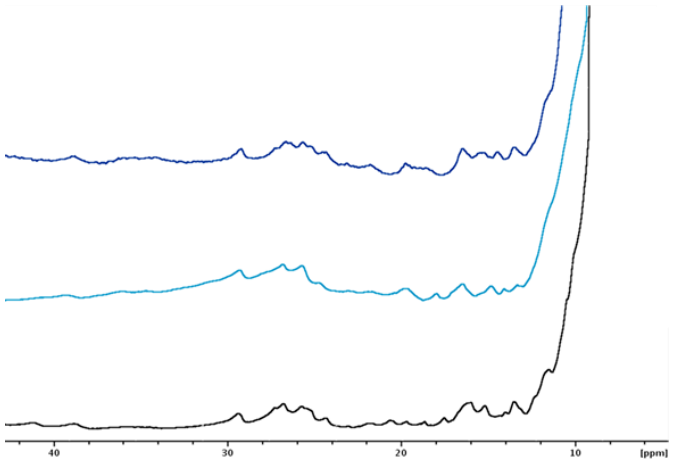
The possibility of a continuous feed with anaerobic sludge resulting from methanogenic anaerobic digesters, will reduce or even mitigate the need of an external energy source to maintain the continuous operation of MFC and thus the production of clean water.

However, towards the practical application of these devices it is crucial to understand the mechanisms that underlies the EET transfer in thermophiles. The comprehensive knowledge of this process will lead to the identification of the rate limiting step of this process which will ultimately allow the customized design of METs.

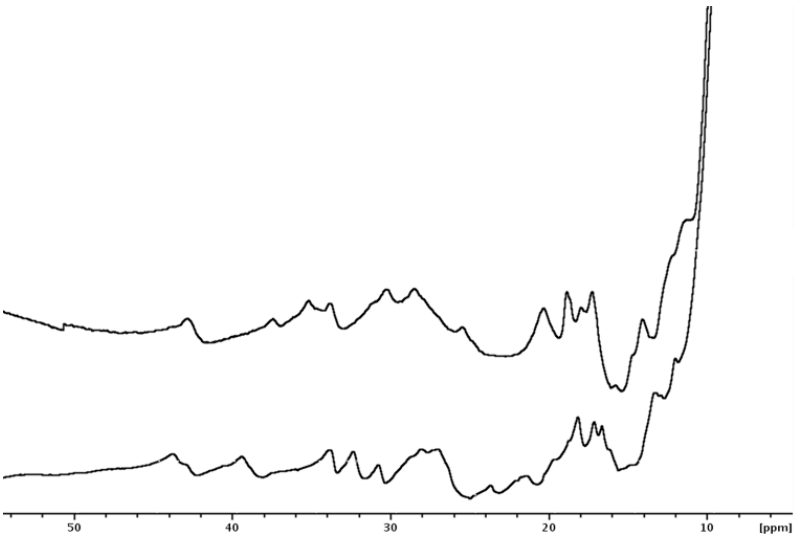
Considering their higher potential for current generation and wastewater treatment further research in thermophiles are required in order to make

the most of future applicability of this promising class of bacteria in industrial process.

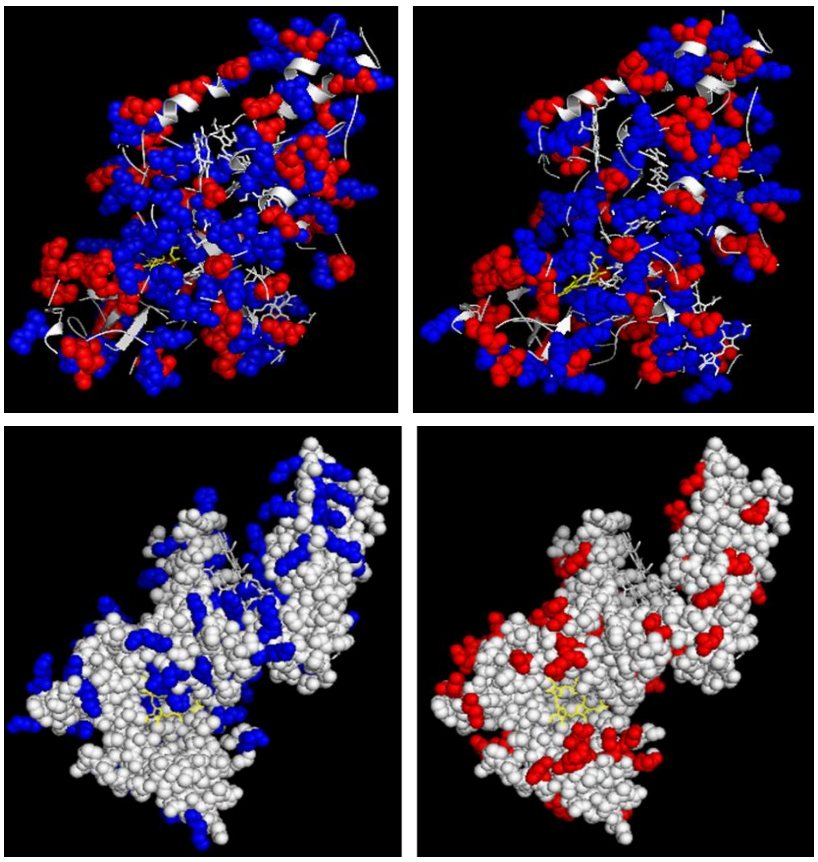
SUPPORTING INFORMATION



S.I Figure V-1: NMR spectra of TherJR_0333: dark blue– TherJR_0333 w/o detergent; light blue – TherJR_0333 with Na-cholate; black– TherJR_0333 with 0.05% DDM at 25ºC



S.I Figure V-2: NMR spectra of OcwA: red – 25ºC; blue – 55ºC



S.I Figure V-3: Structure of OcwA– surface charge distribution red – negative charges; blue – positive charges, white – polar residues

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